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OAK RIDGE NATIONAL LABORATORY

MARTIN MARIETTA

BIOLOGY DIVISION PROGRESS REPORT

For Period of

October 1, 1988 - September 30, 1989

OPERATED BY
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BIOLOGY DIVISION PROGRESS REPORT

For Period of October 1, 1988 - September 30, 1989

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Fred C. Hartman, Director

Section Heads

J. S. Cook, Molecular and Cellular Sciences

R. J. M. Fry, Cancer Biology

R. J. Preston, Human Genetics

L. B. Russell, Mammalian Genetics and Development

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FOREWORD

The Biology Division of the Oak Ridge National Laboratory is one component of the Department of Energy's intramural program in life sciences. Accordingly, about 75% of the Division's total budget is derived from the Department of Energy through its Office of Health and Environmental Research. With respect to experimental biology, the congressionally mandated mission of this Office is to study adverse health effects of energy production and utilization.

Within this stated broad mission, common themes among the research programs of the Biology Division are interactions of animals, cells, and molecules with their respective environments. Investigations focus on genetic and somatic effects of radiation and chemicals. Goals include identification and quantification of these effects, elucidation of pathways by which the effects are expressed, assessment of risks associated with radiation and chemical exposures, and establishment of strategies for extrapolation of risk data from animals to humans. Concurrent basic studies in genetics, biochemistry, molecular biology, and cell biology illuminate normal life processes as prerequisites to comprehending mutagenic and carcinogenic effects of environmental agents.

Research grants and contracts from agencies other than DOE, secured through the initiatives of principal investigators, comprise the remaining portion of the funding base for the Biology Division. Collectively, these grants complement and enhance the DOE-supported activities and provide positions for students, postdoctoral investigators, and research associates, who contribute enormously to the Division's total research efforts.

The premier challenge to the Division for the past decade has been to maintain the diversity and unassailable quality of research programs, despite chronic budgetary erosion with consequential hardships, as required to adapt to evolving needs of sponsors and society. Shifts in emphases that have occurred recently are reflected by infusion of molecular biology expertise into the Mammalian Genetics Program, by further integration of molecular biology and biochemistry under the theme of Protein Engineering, and by initiatives concerning hazards of radon exposure. During the present reporting period, the Division received new funding through DOE's Human Genome Program to pursue novel strategies for sequencing DNA.

This Progress Report is intended to provide both broad perspectives of the Division's research programs and synopses of recent achievements. Readers are invited to contact individual principal investigators for more detailed information, including reprints of publications.

Fred C. Hartman

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RESEARCH ACTIVITIES

Mammalian Genetics and Development Section

Section Overview - L. B. Russell

The Section's recently added strength in molecular biology has provided new dimensions for the continuing research in basic genetics and mammalian germ-cell mutagenesis. Mutations that are generated in mutagenesis experiments and propagated in breeding stocks are furnishing highly valuable genetic tools for correlated functional/structural analyses of selected genomic regions. In turn, molecular analysis of mutations can help in defining the action of mutagens. Because the mouse and human genomes contain numerous homologous genes and extended regions of homology, it is hoped that some of our studies will make it possible to assign functions to human DNA sequences that might otherwise be characterized only at a DNA-sequence level. Further, recognizing that the mouse can provide a valuable experimental model system for the study of human genetic disease, part of the Section's effort is devoted to detailed phenotypic studies of certain mutations.

The major theme of Rinchik's Molecular Genetics Group is the correlated physical (DNA-structure) and functional study of selected portions of the mammalian genome. The group is making extensive use of existing and newly-induced deletion mutations to derive detailed molecular maps for a number of regions that jointly encompass 1-2% of the genome. Sets of nested (overlapping) deletions involving marker loci were (and are continuing to be) derived from specific-locus mutation-rate experiments (conducted by other groups in the Section). Molecular access to these regions is achieved by mapping DNA clones to the deletions. Functional maps for some of the regions already exist from our past complementation studies, and are being derived for others. These functional maps are currently being refined by using large deletions as mutation-selection "reagents" in additional mutagenesis experiments with point-mutation inducers. The nested deletions are useful as mapping reagents for locating newly-discovered loci, especially those defined only by lethal mutations, on a genetic map. This year, major advances were made in the molecular/genetic analysis of four regions.

The correlating of molecular structure with function is also an objective of Woychik's Insertional Mutagenesis Group. Transgenic-mouse technology is used to generate insertional mutations, which are useful genetic reagents because the

mutant locus is "tagged" with exogenous DNA and can be readily cloned. Ultimately, it is possible to clone and characterize the structure and expression of the corresponding wild-type copy of the gene. This year, the group produced 200 different lines of transgenic mice, which are being bred to generate homozygotes. A variety of phenotypes and associated mutations (including one possibly associated with a homeobox region) are being studied anatomically and developmentally, and several are being analyzed molecularly. The group is also using probes derived from transgenic mice for molecular characterization of agent-induced mutations.

Recent advances toward correlating physical and functional maps owe much to the existence and genetic analysis of arrays of mutations involving loci marked in the specific-locus test. The value of having preserved mutant mouse stocks has thus become apparent from the molecular studies that utilize deletions and other chromosomal rearrangements to such great advantage. Consequently, the Genetic Analysis group continues its efforts to genetically characterize several specific regions of the mouse genome. Complementation analyses, cytogenetic banding of chromosomes, recombinational studies, deletion mapping, and embryological analyses are components of these genetic characterizations. The Group also performs genetic and cytogenetic analyses of mutations that are continually being generated in mutagenesis studies, thus supplying the information on which to base conclusions concerning the influence of various factors on the nature of induced mutations. Knowledge gained from genetic and molecular analyses of specific loci and associated regions, especially that derived from achieving complete ablation of loci in certain complementing combinations, now allows us to classify induced specific-locus mutations, merely on the basis of their phenotype, into large (multilocus) and other lesions, thus adding qualitative to the quantitative capabilities of the specific-locus test.

A relatively new area of the Section's program is the use of mutant mouse stocks for the investigation of basic biological processes and as models for human developmental anomalies or genetic disorders. Godfrey's detailed clinical, physiologic and immunologic analyses of the mutant *scurfy*, for example, has revealed a heritable effect in the thymic microenvironment that shapes the developing immune system; this mutant will become a useful model for certain immune-system-mediated diseases in humans. In other studies, certain translocations that produce relatively late-surviving unbalanced segregants are being used as dependable sources of specific malformations (models of human congenital anomalies); these are being analyzed in combined genetic, cytogenetic, anatomical and molecular studies. Work also continues on dominant skeletal mutations, several of which have been found to closely mimic specific human genetic disorders.

Not only have studies by the Mouse Mutagenesis Group continued to provide highly valuable genetic tools for molecular studies (see reports by Rinchik and Woychik groups), but they have served to discover mutagens (and to define mutagenesis protocols) for "manufacturing" specific, desired types of mutations. Chlorambucil, applied to the most responsive germ-cell stage, has turned out to be more effective than X rays in inducing deletions and other chromosome rearrangements, and should thus provide the means for inducing such mutations throughout the genome, allowing high-efficiency recovery of developmentally significant variants at many loci. For the "manufacture" of predominantly *small intragenic* mutations, which are needed for functional analyses of specific chromosomal regions, our protocol of ENU dose repetition in stem-cell spermatogonia has proved to be of the highest utility.

The recent considerable expansion in the specific-locus-mutation data base has made possible an examination of the effects of germ-cell stage on both quantity of mutation yield and nature of mutations. Several patterns were identified according to the male germ-cell stage(s) at which a given chemical elicits its maximum response. The *nature* of mutations (as revealed by lesion size and distribution of mutations among the marked loci) appears to be determined primarily by the mutagenized germ-cell stage, rather than by the identity of the chemical. Mutagenesis studies have also revealed several female-specific mutagens, and have led to the suggestion that the relatively diffuse state of oocyte chromosomes may permit attack by certain chemicals (e.g., intercalating agents) that are ineffective in the condensed chromatin of postmeiotic stages of the male.

Experiments with zygotes have continued to yield exciting findings. Generoso had shown earlier that exposure to ethylene oxide, near the time of sperm entry or shortly afterwards, resulted in high frequencies of miscellaneous fetal malformations and of mid- and late-gestation death, effects that are not seen following any preconception or embryonic exposures to any agent. Several chemicals have now been tested in the zygote system, and those that form N-7 guanine adducts in DNA have been positive. The Chromosomal Damage Group has shown that the fetal anomalies are not mediated by the maternal environment or caused by chromosome breakage or missegregation. This year, the group investigated the possibility that certain chemicals may cause new integrations of proviral elements in the genome; preliminary results with treated zygotes have been encouraging.

The sensitivity of zygotes to the induction of gene mutations was investigated with N-ethyl-N-nitrosourea (ENU) by the Mouse Mutagenesis Group. When mutations are scored in the maternal genome of the zygote, we find a rate that is almost an order of magnitude higher than that induced in spermatogonia,

and a very high frequency of mosaics among the mutants. Since somatic/germline mosaics can be excellent tools for developmental studies, the ENU exposure of early zygotes may provide a method for producing such biological reagents. When mutations were scored in the paternal genome of the zygote, the incidence was considerably lower. A study by Selby of mice born and weaned following zygotic ENU exposure demonstrated the induction of high incidences of dominant skeletal mutations.

The Germ-Cell DNA/Proteins Group this year applied three areas of germ-cell investigation (molecular dosimetry, *in vivo* DNA repair, and measurement of DNA strand breakage) to the study of acrylamide monomer (AA), a chemical that had been thought to be mutagenic only in mammalian germ cells. These studies indicated that for AA, sperm protamine -- rather than DNA -- is the critical target for induction of heritable chromosome aberrations. AA is also interesting in that molecular binding, as well as UDS response are considerably delayed, suggesting that AA is first metabolized to a more reactive chemical. The work also illustrated the powerful role of repair on transmission of genetic damage. In germ-cell stages in which repair does not occur, the stage-related pattern of AA-induced DNA strand breakage paralleled that for heritable genetic effects; however, DNA breaks were also found to be induced in earlier (repair-capable) stages from which *no* chromosome aberrations are transmitted, presumably as a result of repair. The alkaline elution techniques developed by Sega for measuring DNA strand breaks in germ cells after exposure to differently acting agents may eventually prove applicable for monitoring DNA damage in human sperm.

While each group of the Mammalian Genetics and Development Section has its own active program, there are numerous interactions between groups. A prime example is the close interplay between the genetic characterization of mutations in certain regions of the genome and the molecular analyses of these same regions (Russell and Rinchik groups, Russell and Woychik groups). Probes developed in insertional-mutagenesis studies are being used to analyze mutations generated in conventional mutagenesis experiments, as in the case of a translocation associated with limb defect (Woychik and Generoso groups). Functions associated with specific genomic regions are defined in detail by clinical studies (Godfrey interactions with Rinchik and Woychik). Methods developed for the detection of dominant skeletal mutations are being applied to search for mutations in transgenic lines of mice (Selby and Woychik groups). Patterns of adduct binding to DNA and/or protamines in germ cells are being correlated with patterns of induction of transmissible chromosomal damage (Sega and Generoso groups). Several other examples could be cited. Collaborative studies with other laboratories are mentioned in individual reports.

An interactive program to develop new methods in DNA sequencing is being coordinated by Jacobson. This involves three other members of the Division, as well as investigators from other ORNL divisions and an independent laboratory. Jacobson is also interacting with another ORNL division in studying the effects of metals on *Drosophila* DNA and proteins.

MOLECULAR GENETICS AND MOUSE-GENOME STUDIES

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The major interests of the Molecular Genetics Group lie in exploiting spontaneous and induced heritable mutations of the mouse to learn more about the composition and organization of the mammalian genome, as well as to understand better the genetic control of normal and abnormal mammalian development. The continuing, underlying theme of the group's efforts combines basic mouse genetics with the techniques of molecular biology and DNA analysis for the study of both the physical (DNA structure) and functional aspects of mutations. Further, the in-depth study of mutations, along with their corresponding wild-type genes, can provide information useful to the Human Genome Initiative. Many regions of the mouse and human genomes manifest significant homologies, and it is these homologies that allow us to exploit the mouse as a valuable experimental model system for the study of human genetics and human genetic disease.

For almost 40 years, germ-cell specific-locus mutagenesis experiments at ORNL have generated an impressive collection of various agent-induced mutations, many of which are still maintained in breeding stocks. [Moreover, new, more efficient protocols for the production of specific types of mutations are continuing to be described (see below).] These mutations vary widely in degree of phenotypic complexity, in severity-of-effect, and in structure at the DNA level. An important subset is comprised of radiation-induced lethal mutations, which have occurred at several specific regions within the mouse genome. Many of these mutations have been shown to be chromosomal deletions of varying lengths, and we estimate that, in aggregate, they cover approximately 1-2% of the genome. The generation of heritable deletion mutations and their use as biological "reagents" have become the common denominator of the group's interests, which can be summarized as follows: (1) molecular mapping and genetic characterization of genomic regions associated with radiation-induced lethal mutations; (2) high-efficiency, saturation

"point" mutagenesis of these same regions, as a model system for detailed, fine-structure *functional* mapping of large genomic segments; and (3) development of chlorambucil mutagenesis as a method to generate and select heritable deletion mutations throughout the mouse genome.

Molecular Genetics of Radiation-Induced Mutations

Over the past few years, we have concentrated on gaining molecular access to complexes of overlapping, radiation-induced deletion mutations at both the albino (*c*) and pink-eyed deletion (*p*) loci in chromosome 7. This past year has also seen the initiation of genetic and molecular studies on a complex of lethal mutations at the brown (*b*) locus in chromosome 4, and the continuation of genetic and embryologic analyses of a series of dominant, radiation-induced mutations at the Steel (*Sl*) locus in chromosome 10.

The largest of the 37 *c* deletions is 6- to 11-cM in length (perhaps 6-20 million bases of DNA), and the largest of the 43 hypothesized *p* deletions is at least 3 cM in length (perhaps >3 million bases). Genetic analysis of the panel of *c*-region deletions has resulted in a functional map that defines genes important for prenatal, neonatal, and juvenile well-being, proper regulation of a host of metabolic enzymes and blood proteins, male and female fertility, and inner-ear development. Analysis of *p*-region and *b*-region deletions is now yielding similar information on the phenotypes associated with genes in these regions (see also the section on Genetic Analysis).

We have used the panel of *c* deletions to map several DNA probes to this region. These probes were derived from libraries created from flow-sorted or microdissected chromosomes or from the cloning of specific loci, such as the tyrosinase (*c*) gene itself or integration sites of *c*-linked endogenous retroviral sequences. The extensive panel of overlapping deletions is simplifying the ordering of these clones both with respect to other clones and with respect to the gross functional map inferred from the genetic analysis of the deletions.

These probes serve as important access points for diverse areas within the 6-11 cM region corresponding to the largest *c* deletion. Some of the clones can discriminate between members of grossly defined complementation groups, and others are being used in large-fragment DNA analysis to identify distances to deletion breakpoints. We have also been successful in identifying and cloning DNA restriction fragments that carry the breakpoints of several of the radiation-induced deletions. The molecular cloning of these fusion fragments will set the stage for molecular analysis of the breakpoint itself, as well as enabling a molecular "jump" into other linked regions associated with interesting genes

required for development of the implantation embryo, the neonate, and the inner ear in juvenile animals.

A similar analysis is being carried out for lethal *p*-locus mutations, in which several clones have been mapped to the largest *p* deletion. One clone, in fact, maps close to *p* itself, is missing in what we project are some of the smallest *p* deletions, and identifies a polymorphism at the DNA level that is peculiar to chromosomes carrying the old *p* mutation of the mouse fancy. We shall soon initiate large-fragment DNA analysis of these *p* deletions and continue the strategy to identify and clone deletion breakpoint-fusion fragments, which will allow the same type of long-range molecular jumping within the *p*-region deletion complex as we have done within the *c* deletion complex.

Analysis of 24 lethal brown (*b*)-locus mutations with a clone thought to encode the *b* gene itself (in collaboration with I. Jackson, MRC Human Genetics Unit) showed that all were deletions. The same strategies of molecular mapping and genetic analysis are currently being applied to this complex of deletions in Chromosome 4.

Specific-locus mutagenesis tests designed to look for induction of mutations at recessive tester loci, such as those described above, routinely yield a number of *dominant* visible mutations at other loci. We have amassed a collection of dominant mutations at the Steel (*Sl*) locus in chromosome 10. Independent mutations at the *Sl* locus can have variable effects on hematopoiesis, pigment-cell migration, and gametogenesis. Molecular-genetic analysis of the *Sl*-locus region and our large series (>40) of dominant *Sl* mutations can address several general and fundamental questions in mammalian developmental genetics. Some of these questions relate to the basic molecular and functional nature of dominant mutations (the type of genetic alteration that is most important, by far, for meaningful genetic-risk estimation); the existence of, and characterization of, "hotspots" for chromosomal translocations; the nature of cell-environment interactions during development; and the nature(s), mechanism(s), and effect(s) of mutational pleiotropy, in which a single mutation influences more than one (apparently unrelated) developmental pathway.

The genetic analysis of *Sl* mutations is being carried out both by our group and by the Genetic Analysis Group (L. B. Russell). The Oak Ridge *Sl* mutations vary widely in their effects on each of the developmental pathways described above, and at least five of the mutations are associated with translocations involving Chromosome 10. We have evidence that homozygotes for at least seven prenatally lethal, radiation-induced alleles of *Sl* die before the typical "late" (>day 14), macrocytic-anemia-associated lethal period that is characteristic of homozygotes for the original *Sl* allele. Detection of these

early-death phenotypes suggests that these mutants may carry deletions that remove neighboring genes required for normal fetal development to proceed to mid-gestation.

Direct deletion-mapping experiments with an electrophoretic variant at the closely linked (3 $\pm$ 2 cM) Peptidase-2 (*Pep-2*) locus have demonstrated that the seven mutations are not deleted for *Pep-2*. Control crosses, which incorporated the closely linked *Pep-2* marker, rigorously proved that the *Sl/Sl* genotype for these "early-death" alleles is indeed lethal. We are now creating a DNA-mapping resource from these "early-death" alleles balanced opposite *M. spretus* chromosomes 10, as well as from homozygotes for typical late-death, radiation-induced *Sl* alleles to aid in the molecular analysis of this gene and region once molecular probes become available.

High-Efficiency Mutagenesis of Deletion-Associated Regions

During the past year, we completed the pilot of a large-scale mutagenesis experiment designed to "saturate," with presumed point mutations, specific regions of the mouse genome associated with long, radiation-induced deletion mutations (the very regions that are the targets of our molecular-mapping analyses). This experiment was designed (1) to establish an estimate of the minimum number of genes within a defined-length region that are mutable to specific, biologically significant phenotypes; (2) to provide, for several regions of the genome, a *fine-structure functional map*, based on a series of heritable point mutations with characteristic phenotypes, which can subsequently be correlated with a detailed molecular/physical map currently being developed for these same regions; and (3) to provide fundamental genetic, logistical, and statistical information on which to base strategies for subsequent large-scale expansion of the functional maps of mammalian genomes.

The experiment involved a large (6- to 11-cM) deletion of the *c* locus in chromosome 7. A two-generation-cross protocol was utilized that placed highly mutagenized (*N*-ethyl-*N*-nitrosourea-treated), genetically marked chromosomes 7 opposite this large *c* deletion. New recessive mutations (either lethals or viable visibles) mapping to the deleted region of chromosome 7 could be detected in one of the resultant classes of progeny. Further, any new mutation, including lethals and other detrimental variants, could be propagated in a breeding stock derived from another phenotypic class present in the progeny of these crosses.

Testing of 972 such pedigrees detected four new, nonallelic lethal mutations, two allelic mutations of the inner-ear-development locus *shaker 1* (*sh-1*), and two new, allelic "fitness" mutations, which manifest a runting syndrome. Each of these mutations has also been mapped with respect to

breakpoints carried by members of the panel of radiation-induced deletion mutations, thereby rapidly placing these presumed "point" mutations within the gross functional map and the emerging molecular map of this 6- to 11-cM region of chromosome 7. Indeed, one lethal mutation has been positioned between the distal breakpoints of two large distally extending *c* deletions; the imminent cloning of the proximal breakpoints of these deletions with available probes close to *c* will result in the generation of both proximal and distal molecular access points to the region harboring this new lethal point mutation. Gaining these molecular access points will set the stage for the most fine-structure physical/functional mapping exercise yet: the location of a presumed small, intragenic, developmentally significant mutation on a molecular map. Experiments are also under way to gauge whether the new lethal mutations are cell lethals or organismal lethals, as well as to determine the time at which they arrest embryogenesis. Moreover, based on the success of this pilot project, we have expanded the mutagenesis experiment to screen 3500 gametes, with the hope of achieving saturation mutagenesis of this region of the mouse genome.

We anticipate that, in addition to refining the functional maps of genomic regions associated with deletions, these new (presumably) individual-gene mutations will also be important for future use as function-deficient (or function-altered) hosts for receiving segments of cloned, wild-type DNA via transgenic-mouse technology. These types of correction-of-phenotype experiments, if technically feasible, will comprise an important strategy of gene identification for expressed DNA sequences derived from these regions. Furthermore, it is hoped that such high-efficiency mutagenesis strategies will allow the identification of developmentally crucial loci in the mouse genome that might also be found in homologous regions of the human genome. In this way, perhaps some function(s) could be assigned to human DNA sequences that might otherwise be characterized only at a DNA sequence level.

Deletion Mapping Throughout the Mouse Genome

Most of the extant heritable deletion mutations of the mouse have arisen over a period of decades from radiation-mutagenesis experiments, and they are limited to the genomic regions including and surrounding a limited number of recessive marker loci (see above) and a few loci at which dominant mutations are frequently detected. Therefore, currently available deletions cover only a small percentage of the genome. It is becoming increasing evident, however, that heritable, gross chromosomal rearrangements, such as deletions and translocations, can be essential tools in several aspects of genome analysis. As described in the previous sections, molecular access to a specific chromosomal region, and quite often, to a specific gene of unknown product within a region, can be achieved by the use of deletion mutations for the mapping of DNA

clones. Moreover, large deletions can be used as "mutation-selection" reagents in additional rounds of mutagenesis experiments, resulting in the recovery of new, intragenic mutations that can aid in refining a regional functional map. Deletions can be used as mapping reagents for simplifying the process of locating new loci on a genetic map, especially those loci defined only by lethal mutations.

The Mouse Mutagenesis Group (L. B. Russell) last year reported that the chemotherapeutic agent chlorambucil (CHL) could induce a high frequency of (presumably deletion) mutations in certain post-meiotic stages of spermatogenesis. This frequency is perhaps as high as one mutation per 800 per locus per gamete. During this past reporting period, we analyzed DNA from ten primary specific-locus mutants by Southern-blot analysis with probes derived from the marker locus involved in the mutation (two *c*, three *b*, and four *d*, and one *d-se* mutations were tested). This analysis demonstrated that eight of eight mutations tested that arose in post-meiotic stages were deleted for the corresponding probe; deletions could not be detected with available probes in either of two mutations that arose in stem-cell spermatogonia.

Consequently, the high frequency of induction of germline deletion mutations by CHL should make possible the high-efficiency recovery of developmentally significant variants at any locus, throughout the genome, that are directable amenable to first-stage molecular access and analysis. To this end, we have, in collaboration with Drs. W. Frankel and J. Coffin at Tufts University, designed another CHL mutagenesis experiment to generate deletion mutations throughout the mouse genome. This pilot experiment involves the use of already-mapped, endogenous, non-ecotropic retroviral sequences as target sites for the detection of deletions; about 30-40 target loci (integration sites) can be screened per animal by Southern-blot analysis with appropriate probes. The deletions will be identified by loss of paternal RFLPs, which define specific viral integration sites, in the F1 progeny of CHL-treated males. This pilot experiment should rapidly gauge whether the mutation frequency will indeed be high enough for efficient application of this mutagenesis protocol to other loci throughout the genome. We consider the development of CHL mutagenesis extremely important, as its application should result in the generation of a heritable-deletion-mutation resource that can play a central role in both physical- and functional-mapping strategies being considered for the mouse genome.

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MOLECULAR ANALYSIS OF THE MOUSE GENOME UTILIZING PROBES DERIVED FROM TRANSGENIC MICE

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Insertional Mutagenesis in Transgenic Mice

Recent advances in gene cloning and embryo manipulation technologies are making it possible to probe the relationship between the basic structure of the genetic material and complex traits in the whole organism. Of particular interest is the identification of genes that are directly associated with specific forms of human disease. Strategies to physically map and sequence the entire human genome will contribute to this effort, but physical maps and nucleotide sequence alone cannot, in most cases, be used exclusively to define the function of individual genes. On the other hand, genetic approaches, involving the analysis of individual mutations, have historically proved to be an efficient means of establishing the function of genes in several organisms, including mouse and man.

The mutant locus must be clonable with our available technology to be useful to the molecular geneticist. For this reason, insertional mutations in transgenic mice are becoming increasingly useful as genetic reagents because the mutant locus is "tagged" with the exogenously added DNA and can be readily cloned with existing technology. The cloned mutant locus can then be used to generate probes to ultimately clone and characterize the structure and expression of the corresponding wild-type copy of the gene. With this approach, a molecular connection can be made between a complex phenotype in the mutant animal and the expression of the corresponding wild-type gene in the animal.

Insertional mutations can be readily generated in the mouse using the pro-nuclear microinjection transgenic technology. With this approach, cloned DNA fragments, which have been microinjected directly into the male pronucleus of the fertilized egg, will integrate into the host DNA to become a stable heritable part of the genome. Multiple copies of the microinjected DNA fragment most often integrate at a single site within each line of transgenic mice, usually in a head-to-tail configuration. According to present information, site selection for integration appears to be entirely random throughout the host genome. Based on current estimates, in 10-20% of the individual lines of transgenic mice, integration of the exogenously added DNA will interfere with the expression of a

gene within the host genome and will give rise to a detectable phenotype in the whole animal. Most mutations generated in this manner are recessive and give rise to phenotypes ranging from embryonic lethalities to limb deformities. The mutant locus can be cloned by utilizing the exogenously added DNA fragment as a molecular "tag."

We are conducting a large-scale insertional mutagenesis program in transgenic mice utilizing our extensive facilities here within the Mammalian Genetics Section. We produced 200 different lines of transgenic mice this year and are breeding them to generate homozygotes. Each line is being screened in a systematic manner for a variety of phenotypes ranging from embryonic lethalities to skeletal abnormalities. Since this effort involves Southern Blot analysis of approximately 15,000 DNA samples annually, we developed an efficient method for extracting and analyzing this number of DNA samples. The DNA fragment that is being microinjected to generate these lines of mice contains the reporter gene, chloramphenicol acetyl transferase (CAT), partly because this gene can be used as a selectable marker to facilitate the cloning of the sequences flanking the insertion site.

We are presently following a variety of phenotypes that have developed in the transgenic stocks over the past year and have initiated experiments to characterize the mutant locus in these animals. For example, in one line, called Tg737A, transgenic heterozygotes express a complex phenotype associated with polydactyly on all four limbs, severe growth inhibition, scruffy fur during early development, markedly shortened life span, and possibly tooth defects. Homozygous animals die early in development. We observe only partial penetrance of the phenotype in heterozygotes. The flanking sequence has been cloned from this line and the structure of the integration site is being examined. In another line, called Tg370, the heterozygotes are normal, but homozygotes have a characteristic kink at the end of the tail. Skeletal analysis by P. B. Selby revealed a bone structure very similar to that described for the undulated (*un*) mutant. Allelism testing strongly suggests that the insertional mutant is an allele of undulated. Probes from the PAX-1 mouse homeobox gene, which is reported to be associated with the undulated mutation, are being obtained for the molecular analysis of this mutation.

Molecular Analysis of Structural Rearrangements in the Mouse Genome Using Probes from Transgenic Mice

We are using probes derived from transgenic mice for the molecular characterization of other mutations in the mouse, particularly those involving radiation-induced major structural alterations. For example, one such mutation arose in one of Generoso's radiation experiments. In this mutation, new alleles

of limb deformity (*ld*) and agouti (*a*), two loci normally separated by 20 cM on Chromosome 2, arose simultaneously. Cytogenetic analysis by Cacheiro revealed that a segment of Chromosome 17 is inserted interstitially into the distal end of Chromosome 2, resulting in a smaller than normal Chromosome 17 (17^{d}) and a longer form of Chromosome 2 (2^{17}). Comparison of Giemsa-banded metaphase spreads of the 2^{17} chromosome with its normal counterpart revealed a non-homologous interstitial region immediately adjacent and proximal to the inserted section of Chromosome 17. Molecular probes from an insertional mutation in a transgenic mouse allowed us to characterize the altered expression of a complex gene at the *ld* locus and to clone a rearranged segment of this gene from the radiation-induced allele. Analysis of this rearrangement indicated that a region at or near the *a* locus had become juxtaposed with a portion of the *ld* locus on the mutant chromosome. This result suggests that the two new mutations arose by an inversion of a section of Chromosome 2 with DNA breakpoints in the *ld* and *a* loci. Probes from this region at or near the *a* locus are being used to study the molecular biology of this developmentally important region of Chromosome 2.

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GENETIC ANALYSIS

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Genetic and Functional Analyses of Chromosomal Regions Surrounding Specific Loci: Interactions with Molecular Studies

The value of genetically characterized deletions for molecular studies on DNA structure -- and eventually for structure-function relations -- became apparent from our earlier analyses of mutations in the *d-se* (dilute-shortear) region, and from current studies on the *c* region (see section on Molecular Genetics). Our genetic analyses of mutations involving specific loci and the regions surrounding these loci have continued. The value of having preserved mutant stocks generated over decades has become amply apparent, especially in the case of radiation-induced mutations, an appreciable proportion of which

we now know to be deletions. The ongoing genetic characterizations of several specific regions of the mouse genome are closely integrated with molecular studies.

(1) Pink-eye (*p*) region, Chromosome 7. An intercross matrix for 43 radiation-induced *p*-locus mutations (42 of them pre- or postnatally lethal) continues to be compiled for various parameters, and a preliminary complementation map has been constructed. The results indicate that the mutations can be grouped into at least 16 complementation groups, and that in the vicinity of the *p* locus there are probably 6 prenatal-survival and 2 postnatal-survival functional units ("loci"), as well as one or more units for neurological function. An appreciable number of the mutations do not fit a linear map of complexly overlapping deletions and may represent other small rearrangements; they may, alternatively, be indicative of duplications within the *p* region. Karyotype analysis was performed for a *p*-locus mutation, that we have shown to be deleted for neighboring markers *ru-2* and *Ldh-1* [as well as for more than one "lethal" locus, for *Saa* (mouse serum amyloid gene complex), and for 24.2 (an anonymous sequence from human chromosome 11p)]. This deletion lacks the G-dark band 7C and part of the adjacent G-light band 7B5, thus localizing the region cytogenetically.

(2) Agouti (*A*) region, Chromosome 2. This year, we initiated complementation and flanking-marker analysis of *A*-region mutations. We had earlier shown that one of our radiation-induced *A*-locus lethal mutations, a^x, recombines with A^y (lethal yellow) -- formerly considered to be an allele of *A* -- the recombinants being agouti mice that are homozygous viable. Subsequent analyses of several of our stocks revealed the occurrence of rare recombination between A^y and a^t (black-and-tan), and between A^y and *a* (non-agouti), the recombination frequency in each case being of the order of 0.1%. By the use of flanking markers, we showed that A^y is proximal to *A* (*a*, a^t, and a^x). The gene brachypody (*bp*) is 0.3 cM distal to A^y and presumably 0.2 cM distal to *A*. Linda Siracusa (NCI-Frederick Cancer Research Facility), with whom we have collaborated, has mapped the ecotropic murine leukemia provirus, *Emv-15* (which was known to be closely associated with A^y but not causative of the A^y phenotype), to within 0.3 cM of A^y. Our interpretation of recombinational findings indicate a location either proximal to A^y or distal to *A* (*a*, a^t, a^x), but not between the two. *Emv-15* has thus far not provided the means for molecular access to the *A* locus.

Our genetic analyses of *A*-locus mutations involve six agent-induced lethals, as well as two gross rearrangements that have segregated completely with *A*-locus phenotypes. The flanking markers A^y and *bp* are also included in the grid of pair-wise combinations. Results to date indicate that at least some

of the mutations are deletions that include the A^y-associated survival function, that another survival function lies distal to *A*, that none of the deletions extend to *bp*, and that the lethal translocation mutation complements all others for lethality, indicating that its lethality is due to a lesion on the other chromosome involved in the rearrangement (Chromosome 13). Specific combinations of *A*-locus mutations have been instrumental in demonstrating that a probe derived from a rearrangement (see report by Insertional Mutagenesis group) represents *A*-locus sequences for which these mutations are deleted.

(3) Dilute-shortear (*d-se*) region, Chromosome 9. In earlier work, our extensive genetic mapping of this region facilitated molecular analysis (with entry via the integrated provirus *Emv-3*) that found the structural (molecular) map to be concordant with the functional (complementation) map. With molecular studies continuing in the laboratory of Jenkins and Copeland as well as our own, we are further refining and enlarging the genetic map (a) by entering additional mutations into pertinent portions of the grid, and (b) by exploring the proximal extent of the deletions. Last year, we found about one-third of the Df(*d se*) mutations, but only one d^{pl} mutation, to be also deleted for *ash*, which lies 1 cM proximal to *d*. This year, we continued testing Df(*ash d*) and Df(*ash d se*) mutations for deletion of *Pk-3* (pyruvate kinase-3), using an electrophoretic variant. Jacobson has found that the variant enzyme may be unstable under the conditions of storage used by him and that his earlier (negative) results might be invalid. We have initiated tests with *sg* (staggerer), a closer flanking marker. Our cytogenetic analyses localized the *d se* region to Giemsa bands 9D or 9E1.

We completed work with *dsu* (dilute suppressor, Chromosome 1), which showed that *dsu* suppresses the pigment phenotype but not the neurological defect or the postnatal lethality associated with some *d*-locus mutations. By using overlapping deletions, we showed that *dsu/dsu* can suppress a phenotype associated with complete ablation of the locus, thus, supporting the view that $+^{dsu}$ may furnish a gene product similar to that associated with $+^{d}$.

(4) Albino (*c*) region, Chromosome 7. The structural and functional map of this region has been greatly refined by work reported by the Molecular Genetics Group. Refinements of the functional map have also come from more detailed definitions of phenotypes through collaborative studies with Terry Magnuson's group (Case Western Reserve University). Embryological analyses, this year extended to 5 Bi-group mutations, indicated that one of the functional units for implantation survival can be subdivided: a gene(s) important for the normal development of extraembryonic ectoderm is located distal to a gene(s) important for development of embryonic ectoderm. Three of the mutations are deleted for the former functional unit, and two others for both. The former Bi group has thus ben subdivided into Bem and Bex-groups.

(5) Steel (*Sl*) Region, Chromosome 10. The *Sl* locus is of major interest in that it controls developmental pathways concerned with erythropoiesis, pigmentation, and germ-cell formation. As part of a long-term study, we have proceeded with our genetic analyses of over 60 presumed *Sl* mutations that have arisen in our laboratory during the past 30 years, about 10% of them spontaneously and the remainder in the progenies of mutagenized mice; over 50 are now proven *Sl* alleles. At least five of these (including one of spontaneous origin) are associated with reciprocal translocations that co-segregate with the phenotype. All five have this year been cytogenetically analyzed in high-resolution G-banded karyotypes. In all of them, one breakpoint is located in the D1.32 region of Chromosome 10, while the second breakpoint has various locations (5E3.2, 12A2, 17B1.2, 18 C1.3, and 16C1.2). The *Sl* locus may be assumed to be located in 10D1.32. Because of the appreciable number of *Sl* translocations and the high frequency of *Sl* mutations in general, it is suggested that this region represents a mutational "hot spot" in the mouse genome.

Genetic and Cytogenetic Analyses of Mutations Resulting from Mutagenesis Experiments

Analysis of heritable mutations, in addition to providing significant insights into the structural and functional composition of the mammalian genome, can also furnish information on the actions of specific mutagens or mutagenesis protocols. This year, we have carried out genetic and cytogenetic analyses of specific-locus mutations induced by chlorambucil (CHL) in various germ-cell stages, and genetic analyses of ethylnitrosourea- (ENU) and methylnitrosourea- (MNU) induced mutations.

CHL is highly mutagenic in postspermatogonial stages, but negative in stem-cell spermatogonia. Of 14 mutations induced by CHL in postspermatogonial stages, 10 were homozygous lethal (including one sublethal) and only two viable (two were untestable). Seven of the lethals involved loci for which knowledge gained from earlier complementation and molecular analyses allowed us to conclude, on the basis of the genetic evidence alone, that multi-locus deletions had been induced. In fact, these seven (and one additional mutation) were shown to be deleted for DNA sequences at or near the marker locus (see report by Molecular Genetics Group). One of the lethals is associated with a cytologically-detectable deletion of three G bands. Reciprocal translocations were cytologically identified in at least four of the mutant lines; only one of these involved the chromosome on which the mutated marker gene is located, and even that one is probably independent of the specific-locus mutation. An additional translocation was found in a mottled variant. Thus, exposure of poststem-cell stages to CHL induces high frequencies of translocations as well as of deletions.

The highest yield of ENU-induced mutations (in contrast to CHL-induced ones) comes from exposed spermatogonial stem cells and differentiating spermatogonia. This year, we completed genetic analyses of 191 such specific-locus mutations and found only four of them to be prenatally lethal (these four were at the *s* locus, the only one of the seven loci for which we cannot yet conclude that lethality proves multi-locus involvement). Moreover, about one-third of the mutations were to an activity allele (rather than to a null), a further indication that small lesions are being induced. Similarly, of 37 mutations induced by MNU in differentiating spermatogonia, only one was prenatally lethal (again, at *s*); almost one-half of the mutations were to an activity allele. By contrast, among six tested mutations induced by ENU or MNU in postmeiotic stages, 50% were prenatally lethal, and all were to null alleles.

Genetic tests were also performed on 11 mutations (including eight overt mosaics) induced by ENU in zygotes (see report of Mouse Mutagenesis group). For at least five of the mutations, we were able to rule out misassortment at the first cleavage division as a source of mosaicism (pointing, instead, to lesions affecting only one DNA strand). None of the specific-locus mutations that could be propagated through the germ line gave evidence of multi-locus involvement. Though the number of mutations is small, the results parallel those for mutations induced by ENU in spermatogonia.

Enzyme Regulation in Mouse Mutants

Jacobson has confirmed an earlier report that the *hph-1* mutant causes a deficiency in GTP cyclohydrolase activity in the liver. Two other laboratories failed to confirm the enzyme deficiency. A four-way collaboration has been initiated to resolve this discrepancy.

The Use of X-Autosome Translocations as Tools in Various Studies

Among the large numbers of chromosome rearrangements that have been recovered by us over several decades of mutagenesis studies with various agents is the world's most extensive series of X-autosome translocation [T(X;A)s] - rare types of rearrangement - which played a major role in our development of the single-active-X-chromosome hypothesis. In the recent past, we have continued to use these as tools in studies of gene inactivation, molecular mapping of the X chromosome, chromosome pairing mechanisms, gene expression, and for other purposes (see last year's report).

This year, T(X;A)s have continued to provide tools for gaining an understanding of the regulation of chromosome recognition and synapsis. Terry Ashley (University of Tennessee), with whom we collaborate, has proposed

that chromosome rearrangements that have both breakpoints in G-light bands exhibit only homologous synapsis and normal crossing over, while those that have one or both breakpoints in or at the border of a G-dark band exhibit nonhomologous synapsis and suppression of crossing over. In synaptonemal-complex analysis of a series of T(X;A)s, we have compared the synaptic (meiotic) behavior of these translocations with their G- and C-band (mitotic) cytogenetics. Recent results from the study of four T(X;7)s with different breakpoints revealed a close correlation between the amount and direction of nonhomologous synapsis and the width and position of the pertinent G-dark band. A double-blind study of two T(X;4)s confirmed that when both breaks are in G-light bands, synapsis is restricted to homology, but that when one break is in a band depicted as "stippled" on the standard G-band map (G-dark), there is nonhomologous synapsis equivalent to the width of this band. An interesting finding for both T(X;4)s was that, during the progression of pachytene, the unpaired portion of the *4X* axis shortens to the length of the *4* with which it is proximally synapsed.

T(X;A)s have provided important tools for our Molecular Genetics group in the derivation of Chromosome-7-region-enriched DNA libraries. The fact that the X^7 chromosome in two of our T(X;7)s is 1.4 times the length of the longest normal chromosome facilitates fluorescence-activated chromosome sorting of the X^7, as well as microdissection and microcloning of the pertinent regions of this chromosome.

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CHARACTERIZATION OF MUTANT MOUSE STOCKS

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The Mammalian Genetics Section possesses a rich collection of mutant mouse stocks. These animals provide a valuable resource for the investigation of basic biological processes and furnish animal models of human disease. Initial characterization of mutant stocks includes clinical observations, gross and microscopic morphology, hematology, clinical chemistry, and appropriate *in vitro* assays. Once the affected organs/organ systems are identified, the animals are scheduled for additional investigation at the Biology Division or made available to collaborators in the necessary fields.

We have concentrated on the X-linked recessive mutation *scurfy*, which causes exfoliative dermatitis, anemia, cachexia, and early death (at 24 days of age, on average). The disease is associated with a lymphohistocytic proliferation in the liver, skin, and lymphoid organs. *Scurfy* disease requires an intact thymus, and can be transmitted by thymic grafts but not by bone marrow precursor cells. These data suggest that *scurfy* if a defect in the thymic microenvironment that shapes the developing immune system. Such defects are postulated as the cause of many immune-system-diseases in humans. *Scurfy* mice should provide a model to test these hypotheses.

A dwarf stock resulting from deletions at the *c* (albino) locus was investigated. No morphologic abnormalities (other than diminished stature) were noted though the animals had a shortened lifespan. A collaborative effort with Dr. Darrell Wilson, Stanford University, revealed sharply decreased levels of insulin-like growth factor (IGF-I) in the sera of dwarf mice. Future studies will examine the relationship of growth hormone, IGF-I, and various hormone-binding proteins in the pathogenesis of this mutation.

Initial evaluation of a transgenic mutant, *gifted*, revealed polydactyly, polycystic kidneys, bile-duct duplication, and marked serum icterus. Biochemical tests are pending. Similarities to human polycystic kidney diseases include bilateral renal involvement, autosomal dominant inheritance, and associated biliary lesions (congenital hepatic fibrosis).

We have also examined a neurologic mutant with hyperactivity, circling, and head shaking behavior. No central-nervous-system lesions were noted. Studies were discontinued when genetic analysis showed the mutation to be an allele of *shaker-1* (inner ear defects). Another neurologic mutant (with an X-linked inheritance) was found to be deficient in central-nervous-system myelin. This lesion is similar to that described in the *jimpy* mutation.

MOUSE MUTAGENESIS

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The Discovery of Highly Mutagenic Chemicals that Induce Primarily Deletions and Other Chromosomal Rearrangements

We have continued to contribute to the ongoing effort to enlarge the data base for mammalian germline mutagenesis results, one objective being the generation of standards against which data from other test systems can be compared in order to assess the degree to which short-term tests are capable of predicting genetic hazards in mammals (and thus presumably in man). Another major objective is to explore whether certain patterns of response might be demonstrable. The specific-locus test, which has gained considerable utility as a result of recent genetic and molecular analyses of the marker loci and surrounding regions (see other sections of this Annual Report) is being used to analyze all spermatogenic stages, from spermatogonial stem cells to mature

spermatozoa. Our protocol includes procedures that also yield accurate comparative *productivity* results, which can be interpreted with regard to presumptive induction of dominant lethals and/or germ-cell cytotoxicity, thus adding these outcomes to gene-mutation-rate data.

Two chemicals studied this year, both of them chemotherapeutic agents, were chlorambucil (CHL) and melphalan (MPL), both of which turned out to be highly mutagenic in certain postspermatogonial stages, but negative in stem-cell spermatogonia. Chlorambucil, in fact, may be the most effective germ-cell mutagen studied to date -- even better than ethylnitrosourea in certain germ-cell stages; thus, from exposure of early spermatids, the yield of mutations after only 10 mg CHL/kg is almost one per 100 offspring (one per 800 loci). Genetic, cytogenetic, and molecular studies of the mutations (see reports by Genetic Analysis and Molecular Genetics groups), indicated that most, if not all, mutations induced by CHL in postspermatogonial stages are deletions of various lengths; the incidence of translocations was also very high. In view of the utility of gross rearrangements -- and particularly of deletions -- for molecular-genetic studies, chlorambucil (which, at the most responsive stage, is more effective than X rays in inducing deletions) provides a valuable new research tool.

The mutagenicity pattern for MPL is similar (but not identical) to that for CHL, and the mutation rate for equimolar doses is roughly equivalent during the period of maximum response. Genetic and molecular analyses of the MPL mutations are now in progress, and preliminary results indicate that at least an appreciable fraction of the mutations are deletions.

Factors That Affect the Nature of Induced Mutations

The fact that the number of potential mutagens is almost infinite provides a strong impetus to search for relationships in patterns of effect. The recent considerable expansion of specific-locus-mutation data has made possible an examination of the effects of germ-cell stage on both quantity of mutation yield and nature of mutations. For chemicals mutagenic in poststem-cell stages, three patterns have been identified according to the stages in which they elicit maximum response: (1) early spermatozoa and late spermatids; (2) *early* spermatids; and (3) differentiating spermatogonia. The majority of chemicals tested fall into Pattern 1. Chemicals that are also mutagenic in stem-cell spermatogonia (the minority) do not preferentially belong to any of these three categories.

For only one chemical (CHL) has an entire set of mutations been analyzed molecularly (see report by Molecular Genetics Group). However, the results of genetic and molecular analyses of genomic regions surrounding six of the

specific-locus markers allow us to conclude that any mutation that causes lethality of homozygotes (in the case of *d*, *pre*natal lethality, specifically) must involve one or more loci in addition to the marked one. Such mutations have been classified as "large lesions" (LL), the remainder as "other lesions" (OL). Analysis of the data base shows that, regardless of the nature of the chemical (Pattern-1, -2, or -3), (a) very few LL mutations are induced in either stem-cell or differentiating spermatogonia, and (b) very high frequencies of LLs are induced in postmeiotic stages. Chemicals that are active in both pre- and postmeiotic stages produce LL or OL mutations depending on cell stage.

The distribution of mutations among the seven marked loci was also examined. For mutations induced in stem-cell spermatogonia, this spectrum was similar for all groups of chemicals. The distribution for differentiating spermatogonia generally resembled this spectrum. Since the frequency of LLs among spermatogonial mutations is very low, this spectrum may be indicative of the relative sizes of the target loci. On the other hand, the spectrum for postmeiotically-induced mutations departs in several ways from that for the other groups.

On the basis of both lesion size and distribution of mutations among loci, we conclude that germ-cell stage, rather than identity of the chemical, is the major determinant of the nature of chemically-induced mutations, and that the distinction is between pre- and postmeiotic stages rather than between stem-cell and poststem-cell stages.

Mutation Induction in Zygotes

Last year, we explored additional aspects of zygote mutagenesis. The first part of the zygote period may be regarded as the final germ-cell stage, since the parental genomes are still separate. We showed some time ago that, at certain intervals after sperm entry, the mouse zygote is extremely sensitive to radiation-induced clastogenic damage. More recently, W. M. Generoso of our section has demonstrated the high sensitivity of certain zygote stages to chemical induction of varied congenital anomalies (see report by Chromosomal Damage Group). Two years ago, we initiated a study on the sensitivity of zygotes to the induction of gene mutations, using ethylnitrosourea, ENU (which is known to induce primarily intragenic lesions), in a modified specific-locus test. Zygotes containing one parental genome with a number of recessive markers, and the other parental genome with the corresponding wild-type alleles are exposed to ENU by injecting recently copulated females at one of various intervals after the end of a 45-minute mating period. To date, almost 7000 mice resulting from exposed or control zygotes have been examined.

When the male genome was derived from the multiple-recessive parent, ENU treatment, while causing prenatal death of a large number of individual conceptuses, did not reduce the proportion of copulations resulting in litters. Our initial studies therefore concentrated on this cross, which measures mutation induction in the female genome. Two features of the results were striking: the very high mutation rate and the high proportion of mosaics among mutants. (a) At the most sensitive zygote stage, the mutation rate is almost one order of magnitude greater than that induced by the same dose of ENU in spermatogonial stem cells (in which ENU is considered to be a supermutagen). If ENU is injected at a time when the second meiotic division of oocyte (triggered by sperm entry) is being completed, the mutation rate is 7-8 times higher than when exposure occurs 2.5-3 h later (pronuclear formation). (b) Most of the mutants were mosaics, and, on the basis of what is known about mouse development, some or all of the whole-body mutants too could have developed from mosaic blastocysts. Genetic tests on the overt mosaics confirmed germline involvement in about half of them and ruled out chromosome misassortment at cleavage as a cause of the mosaicism. Some of the results indicated that the mosaics resulted from events affecting one DNA strand of the haploid maternal chromosome, and that these events were primarily intragenic lesions. Since somatic/germline mosaics can provide tools for studies of cell line lineage, gene action, cell selection, and other developmental mechanisms, the ENU treatment of zygotes could provide a method for generating such tools.

Our pilot studies indicated considerably poorer yield following ENU exposure in the reciprocal cross (multiple-recessive genome supplied by mother), which measures mutation induction in the paternal genome of the zygote. Our efforts during the past year have therefore been concentrated on accumulating results from this cross for ENU exposures administered shortly after sperm entry (i.e., the stage we had found to be of maximum effect for the *maternal* genome). We found the number of fertile matings to be reduced by 40% and the litter size by about 50%. It has nevertheless been possible to examine over 1000 weaning-age mice that were exposed to ENU as zygotes and about 700 concurrent controls. The incidence of mutations is less than one-fifth of that induced in the female genome. There is a slight increase in the incidence of small white belly spots, which appear, however, to be a developmental, rather than a genetic effect of the treatment.

Maximizing the Induction of Intragenic Mutations

Various lines of evidence have shown that ENU exposure of mouse stem-cell spermatogonia induces primarily intragenic mutations, at least some of which are single-base-pair changes. For investigations that require such mutations, it is important to use protocols that maximize the yield. We had

demonstrated earlier that, below certain exposure levels, there is repair of ENU-induced lesions in stem-cell DNA. High single doses of ENU, on the other hand (e.g., 250 mg/kg) cause extensive spermatogonial killing and selection-associated mutation-rate decreases below linear extrapolation.

To determine whether doses above the levels at which repair occurs are additive, we exposed males to four fractions of 100 mg/kg each at weekly intervals. Additivity was demonstrated, with the spermatogonial stem-cell mutation rate being higher than 1×10^{-3} per locus, about 2.2 times the rate obtained with a single dose of 250 mg/kg. Because a high incidence of cluster mutations complicated calculations of significance, the experiment was repeated. The significance of the difference between the combined data for the 4×100 mg/kg study and the mutation rate obtained with 250 mg/kg is now very convincing (P, one-tailed = 0.003). The very high rate of induction of presumed intragenic mutations is of great practical value for studies requiring the "manufacture" of such mutations, e.g., our saturation mutagenesis study being done in conjunction with molecular analyses of specific chromosomal regions (see report by Molecular Genetics Group).

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CHROMOSOMAL DAMAGE

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High Frequencies of Fetal Anomalies Produced Subsequent to Mutagen Treatment of Zygotes - A New Phenomenon in Mammalian Mutagenesis

There are three general methods for experimental induction of developmental anomalies in mammals. First, high frequencies of congenital anomalies are generated by exposing the embryo *in utero* to teratogens. The type of anomaly produced is closely related to the stage of exposure, and the highest overall yield of anomalies occurs when exposures are given during the period of major organogenesis, between days 6 and 13 of gestation in mice. Second, certain mutations that arise following pre-mating exposure of parents to mutagens are expressed as congenital anomalies. This class of induced congenital anomalies has been observed only at low frequencies. And third, integration of transgenes (exogenous DNA sequences) into the mouse genome has proved to be a valuable method of producing developmental mutants for basic studies.

We have discovered yet another effective method of inducing congenital anomalies. When female mice are exposed to the mutagens ethylene oxide or ethyl methanesulfonate near the time of fertilization of their eggs or during early stages of zygote development, many of the conceptuses die at mid- or late gestation, and a high proportion of the surviving fetuses have hydropia, limb, tail, and other defects. These characteristic effects were not induced or induced only at very low levels by rays, ethylnitrosourea, and triethylenemelamine. This year we observed that diethyl sulfate, but not methyl methanesulfonate, induced these characteristic effects. Thus, results to date indicate that the chemicals that are effective in inducing these endpoints are those that produce primarily N-7 guanine adducts (either ethyl or hydroxyethyl) in DNA. The effects appear to be caused by direct damage to the zygotes, as shown by reciprocal egg-transfer experiments, but they are not the conventional gene mutations and chromosomal aberrations. Results of a nuclear transfer experiment indicated that exposures of the pronuclei, as well as of the cytoplasm, are necessary for the production of the characteristic defects.

The fetal malformations produced in these studies are generally similar to common human sporadic defects, for which the etiology is usually unknown.

Thus, the new phenomenon is of major interest both in chemical safety evaluation and in studying the etiology of congenital abnormalities. Not only does it raise questions regarding the vulnerability of human zygotes when the mother is exposed to environmental chemicals shortly after conception, but it may provide a model for the class of congenital malformations in humans for which the etiology is still unknown.

Induction of Heritable Integration of Retrotransponsons

About 0.05% of the mouse genome is believed to be made up of endogenous viral sequences. We are investigating the possibility that chemical mutagens may cause new integration of certain proviral elements in the genome. For a variety of reasons, we chose to expose the zygotes of the inbred strain, PL/J, to EMS. PL/J is viremic and normally has ecotropic MuLV at three sites in its genome. New integrations of MuLV were detected by RFLP analysis with at least two restriction enzymes among midgestation fetuses.

Results to date show that a low frequency of animals in this strain have four copies of the MuLV instead of the normal three, that fetuses in the control group with new spontaneous acquisition involved integration at two or more sites during early embryonic multicellular stages, and that EMS treatment of zygotes appears to significantly induce new MuLV integration at single sites at apparently random locations in the genome. These results are encouraging; if confirmed, the phenomenon should be of considerable practical as well as basic significance as a mechanism for *in vivo* chemical mutagenesis.

Chromosomal Imbalance in Malformed Fetuses

Reciprocal translocations are readily induced by certain physical and chemical mutagens in certain germ-cell stages. Most individuals heterozygous for a balanced reciprocal translocation do not, themselves, exhibit an abnormal phenotype other than reduced fertility, which results either from impaired gametogenesis (complete sterility) or from the production of gametes with unbalanced chromosome complements that lead to early embryonic death ("semisterility"). The embryonic death caused by most cases of chromosomal imbalance is so early as to have little or no medical importance. Certain unbalanced segregants, however, live longer and exhibit congenital malformations. Obviously, this class of segregants constitutes a potential medical burden in humans. We have in progress a systematic study of (1) the incidence of heritable translocations that produce this class of segregants, (2) the cytogenetic nature of chromosomal imbalance in malformed fetuses, and (3) the types of meiotic segregation that produce late-surviving unbalanced segregants. We are currently studying 24 methylenebisacrylamide-induced

semisterile translocations. Although this sample is small, it appears that translocations that produce defective fetuses are far more frequent than previously realized. Over one-half (13) of the semisterile males sired litters that had late-dying and/or malformed fetuses. Karyotype analyses completed to date indicate that the rearrangements involved at least one breakpoint that is located close to either the centromere or the telomere, and that malformed fetuses were partially monosomic for a very short terminal chromosome segment and partially trisomic for a segment that can be of various lengths in different translocations.

In addition to being the subjects of fundamental studies, these translocations are a dependable source of specific malformations that can be useful as models of human disorders and can provide material for morphogenetic studies that cannot be done in human subjects. Translocation stocks that produce fetuses with frontonasal dysplasia or exencephaly are under investigation in collaboration with Dr. Kathy Sulik of the University of North Carolina and Drs. Joe Rutledge and Thomas Shepard of the University of Washington.

There is currently an upsurge of interest in mouse mutants associated with developmental defects for studies of abnormal and normal mammalian development, underlying genomic structure, and homologies with man. Chromosomal rearrangements are particularly valuable because the location of chromosomal regions associated with the phenotype are cytogenetically identifiable, which sometimes can provide a convenient starting point for molecular studies. Our systematic collaborative approach combining genetic, cytogenetic, anatomical, and molecular studies on certain stocks such as those described above, should contribute to the understanding of the complex pathway involved in abnormal development.

Female-Specific Chemical Mutagens

Evaluation of the mutagenic hazard of chemicals to humans must take into account the fact that the germ cells of the two sexes undergo very different maturation processes. In humans and in all mammals, the chromosomes in the majority of oocytes of adult females are arrested in a diffuse state in the diplotene stage of meiosis. In adult males, on the other hand, all stages of spermatogenesis are present simultaneously, and in several of them the chromosomes are relatively more condensed than they are in arrested oocytes. Despite these fundamental differences between male and female germ cells, most mutagenesis experiments have concentrated on males, and assessment of genetic risk to female germ cells has, so far, received little attention. We have been finding an increasing number of chemicals that pose genetic risk to female- but not to male-germ cells. One class, in particular, are the intercalating agents.

The chemicals, hycanthone (an anti-schistosomal drug) and adriamycin (a cancer chemotherapeutic drug), induce dominant lethals in female- but not in male-mice. We hypothesize that the reason these chemicals are mutagenic only in female mice is that the oocyte DNA is in a diffuse state, allowing the molecules to intercalate between adjacent base-pairs. The inherent difference in the configuration of chromosomes in germ cells of the two sexes could be the major factor in the differential susceptibility to certain mutagens.

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ORGANISMIC EFFECTS

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Generoso et al. (*Mutat. Res.* **176**: 269-274 and **199**: 175-181) discovered that there are remarkable increases in the incidence of developmental abnormalities in fetuses after early zygotic stages are exposed to any one of several chemicals, including ENU. L. B. Russell et al. (*Proc. Natl. Acad. Sci. USA* **85**: 9167-9170) found that ENU induces specific-locus mutations when it is administered to zygotes shortly after sperm entry (2.5-3 h after mating). During FY 1989, we followed up on these findings by carrying out a study, in collaboration with W. M. Generoso, on the induction of dominant skeletal mutations after 40 mg/kg of ENU was injected i.p. into females at either 2.5 or 5 h after mating. There was a concurrent control, and parents were randomized among the three groups. Offspring were coded until after their skeletons were classified. Among mice living 3 weeks or longer, the frequencies of offspring with presumed dominant skeletal mutations were 4/108 (3.7%), 22/208 (10.5%),

and 20/156 (12.8%) in the control, 2.5-h, and 5-h groups, respectively. The experimental frequencies were both significantly higher than the control, $P < 0.03$. The average induced frequency (i.e., experimental minus control) of presumed dominant skeletal mutations was 7.8%. First-generation offspring with stunted growth or externally visible abnormalities were mated before their skeletons were prepared to permit a breeding test for some of the presumed dominant skeletal mutations; the progeny collected are now being examined. Since such high frequencies of induced dominant mutations were found by postnatal examination of just one body system, and after only 40 mg/kg of exposure, it seems likely that the high frequency of abnormal fetuses found by Generoso et al., following a 75 mg/kg ENU exposure also resulted from dominant mutation.

The primary emphasis in the study of dominant skeletal mutations is to collect data that can be used to improve estimates of genetic risk from radiation and chemicals by the direct method of genetic risk estimation. The importance of having such data is becoming more apparent as the problems of deriving reasonable estimates of genetic risk by the doubling-dose method are becoming more obvious. In fact, the United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR), starting in 1986, abandoned the application of the doubling-dose method to the genetic disorders of complex etiology, which constitute the overwhelming majority of human genetic disorders. My detailed critical review of the whole area of dominant mutation induction and risk estimation is therefore timely. This review, which deals with skeletal abnormalities, cataracts, externally visible changes, litter-size-reduction, congenital malformations (detected in late pregnancy), stunted growth, shortened life span, tumors, and effects on behavior, identifies gaps in our knowledge and discusses profitable directions for future research.

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GERM-CELL DNA/PROTEINS

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The purpose of our research is to develop an understanding of the molecular processes that lead to the induction of mutations in mammalian germ cells, using the mouse (both males and females) as a model system. A better understanding of these mutational processes at the molecular level is important for establishing reasonable guidelines for estimating human genetic risk from exposure to chemicals and radiations in the environment. It is also a prerequisite for the development of molecular techniques to monitor human germ cells for potential genetic damage. The work can be divided into several interrelated areas of research: (1) *Molecular dosimetry* to measure the amounts and types of interaction between chemical agents and genetically significant targets within the germ cells. This dosimetry gives valuable information on the relationship between exposures of the whole animal to a mutagen and the amount of mutagen actually reaching the germ cells. (2) *In vivo DNA repair* studies that give important information, in a relatively short period of time, on the extent to which a chemical agent is able to reach the DNA of mammalian germ cells and produce "repairable" lesions. Differential DNA-repair capabilities of various germ-cell stages exposed to the same mutagen can also be measured by means of these studies. (3) *Measurement of DNA strand breakage* in the germ cells following mutagen exposure, using alkaline elution techniques to recover broken pieces of DNA.

Molecular Dosimetry

Molecular dosimetry using radioactively labeled chemical mutagens has been a useful method to reveal the molecular events occurring within germ cells after treatment with mutagens. The procedure is very sensitive and can be used to measure binding of chemical agents to germ cells and to germ-cell DNA at exposure levels that are orders of magnitude lower than those necessary to produce a statistically significant genetic effect. Typically, as little as one lesion in 10^8 deoxynucleotides can be detected with this method. In addition to procedures that make use of labeled mutagens, the use of fluorescence spectroscopy for the detection of DNA adducts is being explored.

We continue to find evidence that protamines, as well as DNA, are important molecular targets for chemical attack that may lead to chromosome aberrations. (The sperm protamines of both mouse and man are simple proteins that are intimately associated with DNA and found only in late-spermatid and mature-sperm stages). Recent studies using i.p. injections of [^{14}C]-acrylamide

(AA) showed large increases in the binding of this chemical to late spermatids and early spermatozoa. Alkylation of DNA within these stages was no more than 0.03% of the total sperm-head alkylations. For an exposure of 125 mg AA/kg, the total binding amounted to about 60 million adducts per cell. Within experimental uncertainty, more than 99% of the sperm alkylation could be attributed to binding to protamine. Furthermore, the temporal pattern of AA binding to protamine paralleled the pattern of genetic damage produced by AA in the spermiogenic stages, while the pattern of DNA alkylation showed no such correlation.

As our molecular dosimetry data are combined with other genetic and cytogenetic data for various chemical agents, we will continue to learn more about the relationship between the extent of chemical damage in the germ cells and the amount of genetic damage expected at realistic exposure levels for humans. Furthermore, we will develop a better understanding of important molecular targets within mammalian germ cells.

DNA Repair

DNA repair can occur in a number of spermatogenic stages but not in the most mature ones. The DNA repair response of germ cells to a given chemical mutagen can vary several-fold among different mouse strains. Our observation of DNA repair in mammalian germ cells after mutagen exposure led us to develop a test for unscheduled DNA synthesis (UDS) that has been used for the screening of potential mutagens because it is fast, sensitive, and rather inexpensive.

Recent studies with acrylamide have indicated unequivocally that this chemical is able to produce DNA lesions in early spermatids. However, the DNA repair response is delayed, with the maximum response occurring 6 h after acrylamide exposure. No other chemical studied to date has shown a delayed UDS response of more than 1 h. Chemical dosimetry studies we have carried out with [^{14}C]-acrylamide have also shown a delay in maximum binding to testicular DNA. The pattern of chemical binding paralleled the pattern of the UDS response, with maximum binding to testicular DNA occurring about 6 h after exposure.

The delayed UDS response of germ cells to acrylamide suggests that, *in vivo*, acrylamide may be metabolized to a more reactive chemical which can then interact with germ-cell DNA. It has been postulated that glycidamide, an epoxide derivative of acrylamide, may be formed *in vivo*. We are currently testing glycidamide to determine if it produces DNA damage in mouse germ cells. We already have good evidence that this chemical is, indeed, producing

a UDS response in mouse germ cells, and we are now beginning to study the level of induced UDS in the germ cells at different times after exposure to glycidamide. If this is the ultimate metabolite of acrylamide *in vivo*, then we would expect to see a maximum UDS response in the germ cells shortly after exposure to glycidamide, rather than the delayed UDS response we see with acrylamide.

Alkaline Elution

To further assess the damage produced in mammalian germ cells using model chemicals, we have developed an alkaline elution procedure to measure DNA strand breaks in the germ cells. The procedure involves lysis of the germ cells after treatment with the test chemical, followed by separation of the DNA double helix using a strong base. Any small pieces of single-stranded DNA resulting from breakage by the test chemical will rapidly pass through a filter, while normal-sized DNA will take much longer to pass through the same filter.

DNA breakage in spermiogenic stages of the mouse was studied after exposure to acrylamide (AA), using this alkaline elution technique. At daily intervals over a 3-week period following i.p. injection of 100 mg AA/kg, mature spermatozoa were recovered from treated ([^{3}H]dThd-labeled) and control ([^{14}C]dThd-labeled) animals and lysed together on polycarbonate filters; the DNA was eluted with a high pH (12.2) buffer. Elution of germ-cell DNA from AA-exposed animals increased (more DNA strand-breaks) in stages sensitive to the genetic effects of AA (late spermatids to early spermatozoa). The stage-related pattern of AA-induced DNA breakage paralleled the pattern of induced genetic damage [M. D. Shelby et al., *Mutat. Res.* **173**: 35-40, 1986] as well as the pattern of sperm alkylation and protamine alkylation found to be produced by AA [G. A. Sega et al., *Mutat. Res.* **216**: 221-230, 1989].

While genetic damage from AA exposure is greatest in stages from late spermatids to early spermatozoa, no genetic damage had been observed in pachytene spermatocytes and early spermatids [M. D. Shelby et al., *Mutat. Res.* **173**: 35-40, 1986]. Therefore, we studied AA-induced DNA breakage directly in pachytene spermatocytes and early spermatids at short times (up to 4 days) after exposure. DNA breakage was clearly detected in these cell stages, with maximum breakage occurring at 1 day after treatment. At later times the breakage gradually decreased, presumably as a result of DNA repair. By the time these cell stages became functional spermatozoa, DNA breaks that could have produced dominant lethal events had apparently been reduced to a level where no genetic effect could be observed.

Our alkaline elution procedure may prove especially useful in measuring accumulation and/or persistence of DNA strand breaks in spermiogenic stages in which no DNA repair is observed after mutagen treatment. It is possible that this method of detecting DNA damage in mouse sperm could also prove applicable for monitoring DNA damage in human sperm.

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NEW METHODS IN SEQUENCING THE MOUSE AND HUMAN GENOME

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One new method for DNA sequencing has been designed so that the rate of sequence determination will be 10-100 times faster than conventional techniques that use radioisotopic or fluorescent labels. The detection of DNA fragments in either the Sanger or Maxam-Gilbert method necessitates locating the DNA after gel electrophoresis; this will be accomplished with resonance ionization mass spectrometry so that many elements and all their stable isotopes can be identified. This will make over 50 labels available that can be used simultaneously in sequencing gels. The chemistry has been developed to use Fe and Sn as labels, and methods to use the rare earths are also being developed. The labels have been shown to be compatible in the molecular biological processes and to be detectable with the mass spectrometer.

Another method for DNA sequencing that is receiving wide attention is scanning tunneling microscopy (STM). The ability to sequence single DNA molecules is an attractive feature of this procedure. Using the Nanoscope I, a commercial STM instrument, the structure of tobacco mosaic virus was examined as a model for sub-chromosome-type particles. We then turned to DNA. In the plasmid PBR332, structures were observed that have definite helical properties. Our goals are to establish procedures for routine sample preparation, to define DNA structure under different conditions, to examine sequencing methods and to evaluate the structure that results from the hybridization of oligonucleotides or the binding of specific proteins to DNA.

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EFFECTS OF TOXIC METALS AND RADIATION ON DNA AND PROTEINS

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The Q(+) forms of tRNA in Drosophila are normally present but at much smaller levels than the Q(-) forms in the young adult. Some years ago we reported that Cd^{++} in the media causes the Q(+) tRNAs to be the predominate form. Now we find that free queuine [(the substance that replaces guanine to give "Q(+)" tRNA)] can protect Drosophila from the toxic effects of cadmium. This introduces a new role for queuine. This study also demonstrated that, like mice, Drosophila are incapable of synthesizing queuine but must obtain it from the diet.

The study of chemical mechanisms of radiation damage to peptides and nucleic acids is progressing. The study on glycylglycine is nearing completion and has provided a confirmation of the Monte Carlo model. The nucleic acid aspect of this study is ready to commence.

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Human Genetics Section

Section Overview - R. J. Preston

The activities of the Section are integrated to provide a composite picture of the potential genetic and somatic effects of radiation and chemical agents to man. This goal is addressed by a research program that is designed to provide an understanding of normal cellular processes (e.g., DNA replication, gene expression) and the consequences of perturbations of these. While this goal is the one that we have had for some time, we continue to utilize new approaches to reach it, so that we take advantage of new techniques developed in our own laboratories or elsewhere.

Recent advances in the area of molecular genetics have made it possible to take an intimate look at the genotype of cells. It is now quite feasible, for example, to characterize a gene mutation at the DNA level by using recombinant DNA cloning techniques and DNA sequencing. Similarly it is possible to determine (1) the specific types of DNA damage that can be induced by radiation or chemical agents; (2) how these different types of damage can be repaired by enzymes in the cell; and (3) the consequences of misrepair or failure to repair damage on the genotype and phenotype of cells, organs, and whole organisms. Such studies will clarify the potential for adverse health effects from human exposure to radiation or chemicals.

In the Human Genetics Section we are studying the steps from induced DNA damage to mutant phenotype, using mouse models and human cells in culture. In addition, we are utilizing other model systems (e.g., hypotrichous ciliated protozoa and *Drosophila*) to identify and characterize genes that are involved in normal cellular processes such as replication and metabolism. Some examples of our current research projects are presented in this overview to illustrate our experimental approaches for addressing these issues; more detailed descriptions can be found in the individual research reports that follow.

(1) A mouse model of sickle cell disease is being developed. Ethylnitrosourea-induced mutations at the alpha- and beta-globin genes that affect the oxygen association-dissociation properties of mouse hemoglobin, together with high-level expression of human globin genes in transgenic mice, provide the bases for developing this mouse model. It is anticipated that such a model system will help in a better understanding of the pathophysiology of the sickle cell disease.

(2) Several studies have indicated that chromosomal alterations are produced by a recombination process, providing an explanation of how specific aberrations might be produced. For example, the frequency of X-ray-induced chromosome interchanges is considerably increased in cells containing chlorodeoxyuridine-substituted DNA. Replication during the repair of induced DNA damage results in a much higher probability of misrepair when the template contains CldU. Also, interchanges induced by restriction enzymes that induce blunt-end double strand breaks require DNA synthesis (repair replication), and it appears that such interchanges can be produced when only one of the two chromosomes involved is cut by a restriction enzyme.

(3) Ciliated protozoa contain a transcriptionally-active macronucleus and an inactive micronucleus. The replication of the macronucleus is localized to a specific replication band (RB) that migrates along the nucleus. By using a macronuclear DNA expression library, DNA clones have been isolated that code for RB-specific proteins. PCNA/cyclin, an accessory protein for DNA polymerase δ, has also been localized to active RBs.

(4) Insecticide resistance in *Drosophila* has been related to a subset of cytochrome P450s. Monoclonal antibodies to this P450 subset have been used to isolate cDNA clones specific for resistance-associated P450. A central fragment of one such clone has been used as a probe to show that the increased amount of resistance-associated P450, and its mRNA, in resistant strains is not a consequence of gene amplification. Instead, the results suggest that the gene in resistant insects is transcriptionally more active because of structural rearrangement.

(5) DNA damages to marine fauna and flora from increased solar UV-B, that is due to ozone depletion, represent a significant concern. The chromatographic estimation of pyrimidine dimers in isolated human DNA is being used for the first time as a biological monitor for ultraviolet light exposure from sunlight in the ocean. Preliminary studies have (a) established a depth/UV-exposure correlation, and (b) shown that laboratory simulation experiments markedly underestimate the DNA damage induced directly by sunlight.

These brief highlights illustrate how it is now possible to study cellular processes at the molecular level, providing the opportunity to understand how specific perturbations can alter normal function. This should allow for a very different approach to obtaining information pertinent to the assessment of risk from exposure to environmental agents. Our current intention is to pursue such approaches with an emphasis on how specific genotypic changes can affect phenotype.

MOLECULAR CYTOGENETICS

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Over the past several years the Molecular Cytogenetics Group has concentrated its efforts on providing information that can be used directly or indirectly to estimate the genetic and carcinogenic risk to man from exposure to radiations and chemical agents. The emphases are in five general areas: (1) to study the mechanism of induction of chromosomal alterations and point mutations; (2) to determine how specific chromosomal alterations might be produced or selected; (3) to investigate the role of such specific alterations in tumor initiation or progression; (4) to study the role of DNA repair fidelity on the nature and spectrum of chromosomal and mutational changes; and (5) to isolate and characterize human DNA repair genes. Current research in these areas is presented in the following sections. In addition, we describe a separate project on the isolation and characterization of a P450 gene in *Drosophila* that is involved in the development of insecticide resistance. The information that we have gathered in this study is being incorporated into other programs.

Mechanisms of Induction of Chromosome Aberrations

We have used restriction endonucleases (RE) as a model system for studying the mechanism of induction of chromosome aberrations. RE produce specific types of DNA damage (blunt- or cohesive-end double-strand breaks) in specific DNA sequences (recognition site). We originally used osmolytic shock to introduce RE into rodent or human cell lines, but are currently utilizing electroporation. This latter method allows for the use of much lower concentrations of RE, but most importantly results in a Poisson distribution of induced aberrations.

We have shown that all RE so far tested produce chromosome aberrations and that the frequency of aberrations is dependent upon calculated cutting frequency and not upon type of double-strand break. The frequency of RE-induced aberrations is increased by inhibitors of repair resynthesis suggesting that a recombination process is involved in the production of chromosome aberrations. We have attempted to address this possibility by two approaches.

In the first approach, we have taken advantage of a previous observation made in our laboratory, namely, that the frequency of sister chromatid exchanges (SCE) is about five times higher in cells grown in chlorodeoxyuridine (CldU) than in cells grown in bromodeoxyuridine (BrdU), and that this increase is due to an increased misreplication of a CldU-substituted template rather than to errors of incorporation of the halogenated base. We reasoned that if X-ray-induced aberrations were the result of a recombination process, then a higher frequency of interchanges would be observed in cells grown in CldU compared to that in cells grown in BrdU. This was shown to be the case. Although the frequency of X-ray-induced deletions was higher in BrdU-substituted cells, indicating a greater sensitivity of BrdU-substituted DNA than CldU-substituted DNA, the frequency of interchanges was considerably higher in CldU-substituted cells. Subsequent experiments have shown that a similar result was obtained for RE-induced aberrations. This indicates that the misrepair of double-strand breaks induced by RE involves a resynthesis step. These studies support the hypothesis that a recombination process is involved in aberration production.

Our second approach to addressing this hypothesis takes advantage of our observation that the RE Dra 1 can produce aberrations in cells that contain non-halogen substituted DNA, or hemi-substituted DNA, but not in cells that contain DNA that is fully substituted with BrdU. If chromosome interchanges are produced by a recombination process initiated by a single DNA double-strand break, then the frequency of interchanges induced by Dra 1 should be equivalent in cells in which all chromosomes contain non-substituted DNA and in cells whose chromosomes have one hemi-substituted chromatid and one fully BrdU-substituted chromatid. In addition, interchanges would involve chromatids that are hemi-substituted (cut by Dra 1) and chromatids that are fully substituted (uncut by Dra 1). Preliminary results suggest that this is indeed the case. Additional experiments are needed before we can draw definitive conclusions about the role of recombination in the production of chromosome aberrations.

Characterization of Chinese Hamster Radiation-Sensitive Mutant Cells and Their Use for Isolating Human DNA Repair Genes

We have initiated studies to utilize X-ray-sensitive mutants of Chinese hamster cells for understanding the mechanism of chromosome aberration induction, the defect in ataxia telangiestasia (AT) cells, and for isolating and characterizing human DNA repair genes. We reasoned that if an excision repair process was involved in the production of chromosome aberrations (by misrepair), and if one (or more) step in this process was defective in AT cells, then mutants that were resistant to cytosine arabinoside (ara-C), an inhibitor of DNA polymerase α, would be X-ray sensitive and also have characteristics of AT cells. In addition, this would be a simple method for selecting X-ray-sensitive

mutants, since ara-C resistance is a "positive" phenotype that can be readily selected, whereas X ray sensitivity is a "negative" phenotype that would require replica plating for selection. Our initial studies proved that our hypothesis was correct; several ara-C resistant Chinese hamster cell mutants were selected and all were more sensitive to killing by X rays than the parent cell. However, the difference in sensitivity was not as great as that between AT and normal human fibroblasts. A second selection at a higher concentration of ara-C (1×10^{-3} M) produced a mutant that was considerably more X ray-sensitive, very similar to AT vs. normal cells. Several studies have indicated that this mutant has the characteristics of AT cells. If normal human cells are X-irradiated and incubated with ara-C for 3 h, their sensitivity to killing is equivalent to that of AT cells. However, if AT cells are treated in the same way their sensitivity is the same as with X rays alone. This suggests that ara-C can inhibit repair in normal cells thereby increasing their sensitivity to X rays, whereas AT cells have a defect that mimics ara-C inhibition of repair, quite possibly an altered polymerase. A remarkable similarity was observed with the ara-C resistant mutant: with normal CHO cells the sensitivity to killing by X rays was enhanced by incubation with ara-C, such that the sensitivity was equal to that from X rays alone. The sensitivity of the mutant was unchanged when treated with X rays plus ara-C compared to X rays alone.

The induction of chromosome aberrations by X rays was also remarkably similar in AT cells and the ara-C resistant mutant. The frequency of X-ray-induced chromosome aberrations was about twice as high in AT fibroblasts irradiated in G_2 compared to normal cells. When cells were incubated with ara-C after X-irradiation, the aberration frequency in normal fibroblasts was increased to the level in AT cells, whereas the frequency in AT cells remained unchanged. Exactly the same result was obtained with the ara-C resistant mutant and normal CHO cells -- the mutant was unaffected by ara-C incubation after X rays, and the normal cells became much more sensitive. We appear to have a model system for studying the mechanism of induction of chromosome aberrations and hopefully for determining the defect in AT cells. In order to do this, it is first necessary to determine if the ara-C mutant and AT cells can or cannot complement one another, i.e., is the defect the same in the two mutants. In order to do this, hybrid cells have to be produced and their X-ray sensitivity determined. We have selected additional markers in the ara-C resistant mutant cell such that it is now $hgprt^-$ and ouabain resistant, allowing it to be readily used for hybrid cell selection. Cell fusion studies have been performed using CHO-K1 $hgprt^-$ or the ara-C resistant $hgprt^-$ cell line with normal human lymphoblastoid or an AT lymphoblastoid cell line. CHO-AT hybrids have the same X-ray sensitivity as the CHO cell, while the ara-C^R-AT hybrid showed no complementation; it was still X-ray sensitive. This result suggests that there might be a common repair deficiency in the ara-C resistant mutant and the AT

cells. Additional experiments will further characterize the defects such that the ara-C^R cells can be used to isolate the human repair gene that reverts the ara-C resistance phenotype and also the AT X-ray sensitivity.

The Role of Specific Chromosome and Gene Alterations in Tumor Induction and/or Progression

In order to study the possible role of specific chromosomal or gene alterations in tumor induction and/or progression, we have utilized several *in vitro/in vivo* tumor systems. We have paid particular attention to the sarcomas because of some rather surprising and very important observations. Silastic discs were implanted into C_3H female mice, and fibrosarcomas developed at the site of these discs. This makes it possible to analyze cells at potentially a very early stage in tumor development. The cells associated with the discs or tumors were cultured and karyotyped and, in addition, direct chromosome preparations were made. In summary, the chromosome numbers varied from 38-350 in all tumors analyzed. There were wide variations in chromosome number between sarcomas, with much less variation within a particular sarcoma. Double minutes (DM) and homogeneously stained regions (HSR), both indicative of gene amplification, were present in many tumors at later stages of development. Consistent chromosomal trends (duplications of chromosomes 10, 16, and 19) were observed in sarcomas from animals that received both the disc and disc plus X rays.

Aneuploidy was a very early change observed in all tumors analyzed. Additional chromosomes were seen even before a histological diagnosis of malignancy could be made with confidence. Attempts were made to relate the presence of DM and/or HSR with oncogene amplification. Southern analysis of 8 tumor lines indicated that there was no association between the presence of DM or HSR and the amplification of c-*myc*, c-H-*ras*, c-*raf*, c-*rel*, c-*mos*, c-*myb*, c-*abl*, c-*erbA*, c-*erbB* or c-*sis*. However, quite remarkably, we did find an association between DM and the amplification of one of the mouse multi-drug resistance (MDR) genes. The significance of this observation, in contrast to all previous reports, is that MDR developed in sarcomas in animals that had not been exposed to any drug or exogenous agent other than the plastic disc. This is an important finding in terms of the development of the resistance of tumors to chemotherapy. We confirmed the drug resistance of these sarcomas by performing cell survival studies on cells with and without DM (i.e., with and without amplification of MDR). The agents tested were methotrexate, cytosine arabinoside, actinomycin D and colchicine. Cells containing DM were considerably more resistant to killing by these agents than were cells without DM over a wide concentration range. There was no relationship between X-ray sensitivity and the presence or absence of DM. Clearly these studies will lead

us to new areas of research, namely the mechanism of induction of MDR, its possible selective advantage to cells grown under apparently non-selective conditions, and the characterization of MDR.

We have also continued our studies of the possible role of specific chromosomal alterations and/or gene amplifications in the initiation and/or progression of mammary tumors using an *in vitro/in vivo* system. We have already described the presence of isochromosome 6 in a high proportion of mammary tumors, and clonal cell lines *in vitro*, and the presence of amplified c-*myc* and its relationship with metastatic potential. All these studies were performed on cells isolated from a single X-irradiated mouse, with comparisons being restricted to a series of clonal lines. In order to determine if we can develop a general hypothesis, it is essential to establish additional cell lines from different animals, and follow karyotypic and oncogene changes *in vitro* in parallel clones.

We have identified two cell lines that were initially non-tumorigenic when injested into cleared mammary fat pads, but subsequently have produced tumors upon reinjection. These cell lines have been cloned and recloned, and three clones from each cell line have been extensively characterized karyotypically by Giemsa banding at every fifth *in vitro* passage. In addition, at the same passages the cells were reinjected into cleared fat pads and any tumors that develop were karyotyped by banding analysis. In summary, particular chromosome changes (structural and numerical) have been observed in several clones, although not necessarily the same ones in each clone, and the same alteration has been observed in tumors. Of particular interest is a very small chromosome (centromere plus light band-identification therefore not possible) that appeared in one clone of one cell line at the same time as the clone became tumorigenic. This same abnormal chromosome was present in tumors that developed upon reinjection. Of course, this does imply an association between the abnormal chromosome and tumor development, but it does provide an exciting avenue to pursue. These studies are continuing and expanding.

We are also continuing an extensive study of the role of deletions of chromosome 2 in the induction or progression of radiation-induced mouse myelogenous leukemia. We have previously shown that such deletions are observed in the majority of bone marrow and spleen cells of leukemic animals. We have recently been able to culture spleen cells from both normal and leukemic animals, and in collaboration with R.J.M. Fry and L.-Y. Ch'ang have initiated a study to characterize the deleted regions of chromosome 2 that could be of significance in the development of myelogenous leukemia.

Molecular Biology of Insecticide Resistance

Insects cause large losses in products of agriculture and forestry. They can be vectors for disease-causing organisms that affect both human and livestock populations. Our capacity to control insect proliferation is rapidly diminishing because they develop resistance to the toxic effects of available insecticides. It has been a common practice to try and overcome resistance by applying more, and more toxic, insecticides. This practice is adversely affecting the health and welfare of man and the environment. An understanding of the molecular mechanisms of resistance could be useful in developing strategies to counter the problems associated with insecticide resistance.

Toward the goal of understanding resistance we have developed a model system in *Drosophila melanogaster*. A subset of cytochrome P450 was shown to be associated with resistance to a variety of insecticides. This subset is expressed in resistant strains at levels 20-100 times that in susceptible strains. Within the period of this report, we used monoclonal antibodies to isolate cDNA clones specific for resistance-associated P450. Partial DNA sequence analysis of such a clone showed structural features common to P450s characterized by others. A central fragment of this clone was used to probe a Northern blot of polyA RNA from a susceptible and resistant pair of strains. The amount of resistance-associated P450-specific mRNA in the resistant strain was more than 20 times higher than in the susceptible strain. Also, the mRNA in the susceptible strain was 100-200 bp longer than that in the resistant strain. Southern blot analysis of DNA from the two strains, using the cloned probe, showed size differences in the major fragments produced by at least three out of five restriction enzymes used. This analysis gave no indication that the P450 gene is amplified in the resistant strain.

Taken together, our current results indicate that the resistance-associated P450 gene in susceptible strains is in a transcriptionally more active form in resistant strains and that the product of this gene, P450, mediates resistance by detoxifying insecticides. Current and further experiments are designed to identify the structural and functional differences between the gene in susceptible and resistant pairs of flies. Because our antibodies to resistance-associated P450 cross react with analogous proteins in other insects, we believe these studies have relevance to economically more important insects and that our antibodies and cDNA clones will be useful probes for studies in those other insects.

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MAMMALIAN BIOCHEMICAL GENETICS

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Our laboratory is studying the genetic and pathophysiological effects of exposing mice to radiation, chemicals, and viruses in order to assess the potential effects of similar exposures to humans. Techniques of molecular biology are being used to characterize radiation and chemically-induced alterations in the organization and structure of genes that change the structures and functions of gene products and often cause poor health. Flow cytometry and immunological methods are being used to determine the cellular basis for the immunodeficiencies observed in beta-thalassemic mice and in B10.F mice that shed endogenous ecotropic viruses. Summaries of our recent results are presented below.

Organization and Regulation of the Hemoglobin Genes in Mice

The alpha globin complex contains three genes (5' x - a1 - a2 3'). The x-globin gene is expressed in a temporal and tissue-specific manner only in the nucleated erythrocytes of visceral yolk sac origin. The a1 and a2 globin genes are expressed in the nucleated erythrocytes and in the nonnucleated erythrocytes that develop in the fetal liver, spleen, and bone marrow. Moreover, relatively more a2 globin is found in nucleated- than in nonnucleated-erythrocytes. The a1 and a2 structural genes and flanking nucleotide sequences from Skive mice have been cloned and are being sequenced to search for unique nucleotide sequences that might be associated with changes in the level of expression of the a1 and a2 genes during development.

Animal Models of Human Hemoglobinopathies

A mouse model for beta-thalassemia was developed from a DBA/2J male mouse in which the 5' beta-dmajor globin gene was deleted spontaneously. We have reported that the peripheral blood hematology of beta-thalassemic mice is clinically similar to that of beta-thalassemia patients. Recurring infections are common in beta-thalassemia patients. We have found that the immune system of beta-thalassemic mice is deficient. Homozygous beta-thalassemic mice are more susceptible to infections by *L. monocytogenes* and *S. typhimurium* than are heterozygous beta-thalassemic mice used as controls. The mitogenic response of spleen cells from homozygous beta-thalassemic mice to Con A and PHA (T-cell mitogens) is 20-50 percent of normal, but response to LPS (B-cell mitogen) is normal. The total number of plaque-forming-cells that develop in response to sheep red cell immunization is also reduced in beta-thalassemic mice.

Beta-thalassemic mice provide an excellent animal model in which to study the cellular basis for the observed deficiencies in immune responses. Flow cytometric analysis (FACS) of splenic lymphocytes has shown that the incidence of IgG+ B-cells is only slightly lower in beta-thalassemic spleens than in spleens from normal mice. However, the incidences of "bright" Thy 1+, CD4+ and CD8+ cells are significantly reduced and the CD4/CD8 ratio is increased in beta-thalassemic splenocytes compared with normal splenocytes. Further studies are needed but we know that iron derived from catabolized hemoglobin accumulates in macrophages of beta-thalassemic mice and human patients. We postulate that excess iron may cause the macrophage to become an ineffective antigen-processing cell, which would result in ineffective antigen presentation and/or disruption of the interleukin cascade that regulates lymphocyte differentiation and function.

A mouse model of sickle cell disease is being developed by taking advantage of ENU-induced mutations at the alpha- and beta-globin genes that affect the oxygen association-dissociation properties of mouse hemoglobin and of newly developed methods for high-level expression of human globin genes in transgenic mice. The variant mouse hemoglobins have amino acid substitutions adjacent to the heme-binding sites and they have a higher affinity for binding oxygen (P_{50} = 24.5) than normal mouse hemoglobin (P_{50} = 40). Mice with the high oxygen-affinity hemoglobin [MHOAH] are being made transgenic for the human alpha-globin and beta-sickle Antilles genes using constructs that also contain hypersensitivity sites located 5' to the human epsilon-globin gene. Transgenic mice that express 50 percent or more HbS Antilles are very likely to exhibit sickle cell disease similar to that expressed in humans who are heterozygous for HbA and HbS Antilles. MHOAH mice are being used because the oxygen association-dissociation property of MHOAH hemoglobin provides a favorable environment for HbS Antilles to become deoxygenated and precipitate. The P_{50} value for HbS Antilles is about 45 mm of Hg. Thus, about 60 percent of the HbS Antilles will be deoxygenated in the venous blood at 40 mm of Hg oxygen tension while only 25 percent of the MHOAH hemoglobin will be deoxygenated at the same oxygen tension. This mouse model of sickle cell anemia will facilitate research on the pathophysiology of sickle cell disease and the development and testing of anti-sickling drugs.

Retrovirus-Induced Immunodeficiency in B10.F Mice

B10.F mice are born with maternally transmitted ecotropic retroviruses. These viruses are lymphotropic and integrate to become proviruses in DNA of lymphatic cells. The effects of the acquired viremia on the immune system of B10.F mice are being studied by quantitating the ability of spleen cells to respond to mitogens (Con A, PHA and LPS) and by FACS analysis of the distribution of lymphocyte subsets in the spleen. Compared with B10, B10.F splenic lymphocytes are less responsive to Con A and PHA (0-50% of normal), but their responses to LPS are equivalent. At six months of age, B10.F splenocytes bind less anti-Thy 1.2 antibody and have fewer CD4+ AND cd8+ T-cells. The spleens of some older B10.F mice have an increased number of Ly-1+ and IgG+ cells, suggesting that viremia activates Ly-1, B-cells. These results suggest that the peripheral T-cell subsets of B10.F mice are disturbed, which may explain why these mice give a reduced response to T-cell mitogens.

This past year we learned that B10.F mice transmit several kinds of ecotropic retroviruses rather than a single kind. Individual retroviruses are being isolated by biological cloning methods and are being characterized by restriction mapping in preparation for future studies on the tissue tropism and oncogenic properties of each kind of virus.

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DNA DAMAGE AND DNA REPAIR

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Human cells contain DNA repair systems responsible for the removal of environmentally-induced DNA lesions which are potentially carcinogenic. These DNA lesions may be sunlight-induced DNA photoproducts or chemical adducts produced by environmental carcinogens such as benzo(*a*)pyrene.

Individuals with the genetic disease xeroderma pigmentosum (XP) have a mutation causing a defect in the DNA repair system. Such individuals suffer from multiple sunlight-induced cancers. Cells from classical XP patients show an inability to excise *cis-syn* cyclobutane pyrimidine dimers from their DNA. As a result of these observations, this pyrimidine dimer has been considered a primary candidate for the ultimate ultraviolet-induced carcinogenic lesion in DNA.

Other XP patients, grouped as XP variants (XPV), do excise *cis-syn* pyrimidine dimers but, nevertheless, sustain multiple solar carcinomas. In XPV patients we found a failure to repair another ultraviolet-induced DNA lesion, which is sensitive to 313 nm irradiation and is detected by DNA molecular weight determinations on alkaline sucrose gradients. This lesion is repaired much more rapidly in normal human cells than is the *cis-syn* pyrimidine dimer. The removal of this lesion is temperature-dependent in normal cells indicating that an enzymatic reaction is involved. Biophysical experiments on the action spectrum

for induction and photolysis suggest that this new lesion may be a 6-4 pyrimidine pyrimidone. Such products are induced most readily at 254 nm and are most photolyzable at 313 nm. The fact that this lesion is rapidly repaired in normal human cells may indicate a major biological significance even though its incidence in DNA after ultraviolet (UV) is much lower than that of the *cis-syn* dimer.

Recent experiments with UV-sensitive Chinese hamster ovary (CHO) cell mutants (collaborative with L H. Thompson of Lawrence Livermore Laboratory) indicate that mutants which do not excise 6-4 products, in an immunological assay for these lesions, also do not remove the 313 sensitive lesion. These experiments provide further evidence that this photo sensitive lesion is the 6-4 pyrimidine pyrimidone.

UV-Sensitive CHO Repair Mutants Bearing Cloned Human Repair Genes

Using a radiochromatographic assay, we have examined *cis-syn* cyclobutane pyrimidine dimer removal after ultraviolet irradiation in cell lines representative of the first six complementation groups of Chinese hamster ovary DNA nucleotide excision repair mutants supplied by L. H. Thompson. AA8, the CHO cell line from which these mutants were derived, consistently showed normal dimer excision for a rodent cell. The mutants uniformly exhibited no significant dimer excision within the limits of determination. Additionally, V-H1, a mutant belonging to complementation group 2 and derived from V79 hamster cells, exhibited no dimer excision. Two UV5-derived transformants that carry the complementing human *ERCC2* repair gene showed a capacity for dimer excision comparable to the AA8 wild-type cells.

DNA as a Dosimeter for Solar UV

DNA can be used as a convenient dosimeter for solar UV, a dosimeter which may directly reflect the biological consequences of increased solar UV-B due to atmospheric ozone depletion. While many different kinds of damage are induced in DNA by UV-light, the most frequent and the most easily quantitated is the cyclobutane pyrimidine dimer. We have examined several parameters affecting the induction of pyrimidine dimers in DNA by solar simulators, including (1) dimer induction in isolated DNA compared to that in intact cells, (2) effect of temperature on dimer formation, (3) effect of different cell types, and (4) irradiation through layers of water. Our results indicate (1) more dimers in isolated DNA than in cells, (2) a marked effect on dimer yield at temperatures between 5° and 60° C (more at 60°), (3) an effect of cell morphology on dimer yield at lower wavelengths, and (4) ~ 70% attenuation of dimer yield by 1 meter of water.

DNA as a Dosimeter for Solar UV-B

The principal attenuator of solar UV-B is stratospheric ozone. There is accumulating evidence indicating that the destruction of stratospheric 0_3, is primarily catalyzed by chlorine molecules from anthropogenic sources. The primary consequence of decreased 0_3 is increased incident solar UV-B (290-320 nm) to the earth's surface. Living organisms contain high concentrations of molecules which absorb greatly in the UV-B: nucleic acids and proteins. Increased UV-B irradiation is expected to have biological consequences, the magnitude of which is at present not known. The DNA molecule is a quantitative and integrating dosimeter for solar UV-B. It will simultaneously monitor all DNA damaging wavelengths and also register the higher magnitude of damages produced by the more biologically active, shorter wavelengths (> 310 nm). It would take many data points, collected over varying time spans, to approach the same results using a UV-B spectrophotometer, yet a laboratory based weighting function would still have to be applied to get biologically meaningful results. We have quantitated solar UV-B induced cyclobutane pyrimidine dimers in standardized DNA solutions and in DNA extracted after exposure of human skin cells to the sun at 36° N (Oak Ridge). One hour of summer time noon sun will produce about 2 million pyrimidine dimers in the DNA of cells growing in tissue culture, or an equivalent amount in DNA in solution.

Existing studies which have assessed UV effects in the ocean on such marine zooplanktonic organisms as fish larvae have focused on biological endpoints such as mortality. In these studies it has been suggested that many of the observed biological effects may be due to UV-induced DNA damages. However, few, if any, direct measurements of these damages have been made. The penetration of solar UV-B radiation into the sea has been measured and modelled. Thus, for example, the depth at which the oceanic attenuation leaves 10% of surface levels can be as much as 16 m in clear oceanic waters. However, these attenuation values are calculated for an assumed biologically effective response function, such as the DNA action spectrum, established from laboratory data. To what depth and in what magnitude actual DNA damage can be demonstrated remains a central, and as yet only partially, answered question.

In collaboration with the National Oceanographic and Atmospheric Administration, we performed experiments with DNA dosimetry in the waters adjacent to Lee Stocking Island, Exumas, Bahamas, with assistance from personnel of the Caribbean Marine Research Center (a NOAA laboratory) located there. Several important findings have emerged from these experiments: (1) The decrease in dimer production is linear (log scale) at shallow depths (0-2 m); thereafter the curve is nonlinear and complex; (2) dimers are induced in significant amounts even at 3 m depth; and (3) there was a great discrepancy

between our laboratory findings, which predicted less than 10% dimers at 1 m depth compared to the surface, and our field data which showed 13% of the surface values at 3 m depth. Thus, we find that our laboratory data markedly underestimates the penetration of DNA damaging solar UV-B radiation in the ocean. The large discrepancy between laboratory and field data in these measurements may be due to bottom reflectance, mirroring effects of surface turbulence, or other factors. In any case, our preliminary data clearly indicate that field measurements are necessary for collecting data applicable to natural systems.

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CHROMOSOME CHEMISTRY

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The major goal of this laboratory is to analyze and understand the macromolecular structure of eukaryotic chromosomes and their relation to DNA packaging, transcription, and replication. Our laboratory employs a wide range of biophysical, biochemical, and ultrastructural techniques to work towards detailed macromolecular models.

Chromatin Structure in the Hypotrichous Ciliated Protozoa

All ciliated protozoa exhibit nuclear, dimorphism, i.e., the existence of a transcriptionally-active macronucleus in the same cytoplasm with an inactive micronucleus. The hypotrichous ciliated protozoa possess two distinct nuclear features that distinguish them from other ciliates: (1) macronuclei consist of a "bag" of high polyploid (ca. 10^4-fold), short (ca. 2-3 kbp), linear DNA molecules

of low sequence complexity -- each fragment probably corresponding to an individual structural gene and its regulating flanking sequences; and (2) macronuclear DNA replication is localized exclusively in a Replication Band (RB) that migrates along the nucleus during S phase. Both of these features are unique in biological systems and offer considerable advantages in the study of nuclear structure and function compared to typical eukaryotic nuclei. During the past year, our research has capitalized upon both of these nuclear features. (1) Macronuclear genomic libraries have been constructed, from which several genes have been cloned and sequenced, including the 5S RNA and polyubiquitin gene. In addition, nucleosome positioning studies of 5S RNA and polyubiquitin minichromosome have been completed. In an effort to study mechanisms of control of macronuclear genetic expression, an intensive investigation of two gene families has been initiated, i.e., the heat shock genes and the inner histone genes. Two HSP 70 genes and two H3 genes have been sequenced. Attempts are also underway to develop an *in vitro* transcription assay for pol III and pol II genes. (2) The replication band has received intensive cytochemical, ultrastructural and physiological study. An *in vitro* assay for DNA synthesis by isolated macronuclei and permeabilized cells was developed. Employing a macronuclear DNA expression library, DNA clones have been retrieved which code for RB-specific proteins. Using a human auto-immune serum with reactivity against PCNA/cyclin (accessory protein for DNA polymerase δ), we have obtained immunocytochemical evidence for PCNA localization in active RBs. This auto-antiserum will be tested against the expression library. Current studies emphasize the identification of genes for DNA polymerases and associated proteins and the monitoring of their expression during the vegetative cell cycle.

Three-Dimensional Reconstruction of Electron Microscope Tomography (EMT)

We have been interested in the 3-D reconstruction of asymmetric organelles, specifically of chromosomal structures during transcription, replication, and higher-order packaging. Most attention has been focused upon a chromosomal region of RNA synthesis, the Balbiani Rings (BR) of Chironomus salivary gland cells. This gene is present on highly polytene chromosomes (ca. 10^4 endoreplicated), and, when active, generates a "puffed" region in the chromosome body. In the electron microscope, electron-dense nascent ribonucleoprotein granules (RNP) can be observed surrounding the chromatin axis. We have emphasized reconstruction of thick sections which cannot be visualized in a conventional, 100 KV, transmission electron microscope. To solve this problem, we have collected data on an intermediate voltage electron microscope (IVEM) as well as an energy filtering microscope (Zeiss 902). Both modes of data collection were shown to be useful and acceptable. In fact, since the method of energy filtration has only recently become commercially available,

its use for imaging thick sections is not universally accepted. We completed a collaborative study with a physicist, C. Colliex (Universite Paris-Sud, Orsas, France), in order to demonstrate the theoretical and qualitative reliability of the method. Current studies on the development of EMT are exploring (1) the interactions of electrons with thick plastic sections and the mechanisms of mass loss; (2) reconstruction algorithms that employ some *a priori* knowledge of the structure of interest; and (3) computer graphics routines for manipulating and modeling 3-D structures.

A collaborative study with Bruce Moyer (Chemistry Division, ORNL) led to the development of an improved DNA-specific stain for the electron microscope. We plan to use this stain in studies to trace the 3-D path of DNA in various states of the cell nucleus.

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Cancer Biology Section

Section Overview - R. J. M. Fry

The Cancer Section is concerned with the biology of cancer at the molecular, cellular, tissue and whole-animal level. To understand the origin and course of cancer entails knowledge about almost all the facets of cellular and tissue biology. Our studies are not confined to the fundamental mechanisms of controls of cell activity and the changes with the process of malignancy but also deal with the estimation of risk of cancer after exposure to radiation.

The cardinal change in the cell that endows the potential of malignancy is in gene expression. The realization that altered expression of gene activity without any specific gene mutation can be an important component of the carcinogenic process has made it all the more important to understand the normal control of gene expression.

The understanding of gene expression and its control is central to the understanding of differentiation and the normal function of cells and thus organs. To unravel the complexities of interaction of agents that influence expression of a specific gene, the gene and its products must be characterized. F. T. Kenney, K.-L. Lee and their coworkers have continued their studies on the molecular mechanisms of the control of gene expression, in particular, the hormonal control of expression.

A multihormonally regulated gene, designated gene 33, which was chosen as a model system for investigation of hormonal regulation, has now been characterized completely. Cells of a rat hepatoma cell line have been transfected with a gene 33-CAT construct. In cells containing such constructs, it was shown that there was a rapid response to insulin. The stage was set for the identification of the DNA sequences that respond to insulin, and the results of these studies are discussed by F. Kenney and coworkers. In fact, this gene 33 provides a convenient way to study how the chain of events, starting with the interaction of a hormone and its receptor, results in the production of a gene specific protein. An intriguing finding has been that the gene encodes two discrete proteins, which of course, raises interesting questions about the conditions that determine which of the proteins is produced.

The synthesis of mRNAs and their protein products is characteristic of active cells and the other side of this coin is the intracellular degradation process. The rates of degradation of mRNAs and proteins are surprisingly disparate and it is not clear why. As a consequence of the very rapid turnover

of some mRNAs, it is as difficult to delineate and identify the intermediate metabolites of degradation as catching a will-of-the-wisp. K.-L. Lee and D. M. Yang have been investigating a solution to this problem of obtaining adequate amounts of rapidly turning over mRNAs and proteins using gene amplification, and they report their initial promising results.

Over the years the approaches taken by the Molecular Genetics of Cancer Group, led by W. K. Yang, in their studies of retroviruses and cancer have steadily evolved. Of late, they have placed some of their effort on investigating the roles of retroviral and retrotransposable gene elements. Instability of the genome is a possible outcome of exposure to various carcinogenic agents and is a characteristic of progression in the carcinogenic process. The possibility is being investigated that mobile genes play a role in the induction of instability and oncogene activation. The capability of developing transgenic mice has been added to the armamentarium of techniques in order to study such aspects as the tissue-specificity of expression of specific oncogenes. Individual constructs that contain the sequence for mouse interleukin-3 and GM-CSF have been prepared, and the founder mice with the constructs have been obtained. These mice will be particularly important to studying the role of these growth factors in radiation leukemogenesis, which has become a focus for our molecular, chromosomal, and whole-animal studies of naturally-occurring and radiation-induced myeloid leukemia. Transgenic mice should prove useful for studying a number of aspects, including the role of certain genes and the regulation of gene function.

Another method of introducing specific genes into cells in order to study their effect on tumorigenesis is to use retroviral vector systems. This has been done using various oncogenes and the coding sequences for certain growth factors.

The studies of S. J. Kennel and his coworkers are concerned with a cell surface protein that is expressed in some abundance by cells of some murine cancers. The surface protein has been identified as a member of the integrin family. An analogous but distinct protein has been found in the cells of human carcinomas. The complex appears to consist of five distinct glycoproteins. Monoclonal antibodies have been developed to the point that it is possible to show that expression in certain carcinomas is restricted to one of the glycoproteins of the complex. Evidence of distinct characteristics of the individual glycoproteins opens up a number of possibilities. There is still a great deal of work to be done to establish the suspected receptor function of the complex.

Two antibodies have been developed that recognize a glycoprotein that is expressed exclusively by the endothelial cells of the normal lung. Many of the methods of imaging of tumors are based on the differential uptake in the tumor of some radiolabeled agent. The availability of monoclonal antibodies to normal endothelial cells has opened up the possibility of negative imaging for diagnostic purposes, and liposomes tagged with these monoclonal antibodies have the potential of localized delivery of drugs. These studies are a satisfying example of the potential of applying fundamental studies to a very practical problem.

The spectrum of the studies of carcinogenesis has to be limited in a section of our size. Such limitations force careful selection of model systems that can be used to answer the questions of interest. Rat tracheal cell models have proven a profitable approach. The fact that *in vivo, in vivo-in vitro* and *in vitro* studies of neoplastic change can all be carried out with these epithelial cells is a great advantage.

Two forms of the tracheal implant model that were developed here are used by A. C. Marchok. The so-called open-ended tracheal implant model has been used to study interactions of different agents. The system makes it possible to expose the tracheal epithelium on multiple occasions to single or multiple agents. There is a concern that industrial and environmental exposures that involve multiple compounds may be more toxic or carcinogenic than studies of the individual components would suggest. Just such a case has been found with combined exposures to carcinogenic polycyclic hydrocarbons and formaldehyde, a very weak carcinogen. A synergistic effect was found.

The study of epithelial cells as they change from normal to neoplastic state and progress to malignancy is possible with the tracheal model systems. Cells can be grown out from the tracheal implants for study at different stages of the carcinogenic process. The ability to sample the cells in the development of neoplasia makes it possible to study the sequential changes in growth control, oncogene activation, and expression. Techniques such as NMR spectroscopy are being used to determine the nature and significance of the change in pyruvate metabolism found in cells altered by carcinogens.

The studies of the Radiation Carcinogenesis Group include the mechanisms of radiation-induced leukemogenesis and the factors determining the development of tumors, as well as the more pragmatic aspects related to radiation risk estimates such as dose rate and radiation quality. The rat tracheal cell systems, the skin, and the mammmary gland cells are the models used to study various aspects of radiation-induced cancer.

Tissues are able to suppress the expression of neoplastically transformed cells in their midst. Cells act as a community and normally the cell-cell contact can affect tumor development. Disruption of this cell-cell communication can eliminate the suppression of altered cells. The evidence is strong that the transforming growth factor Type-β modulates cell proliferation and may be involved in the suppression of tumor development.

Tracheal cells have been used to study a question that was raised originally by whole-animal experiments. There is a reduction in the carcinogenic effect of low-LET radiation when the dose rate is reduced. In contrast, some investigators have reported that lowering the dose rate of neutron irradiation had the opposite effect. Exposures of tracheal cells with very low doses of neutrons at low dose rates did not indicate the so-called inverse dose-rate effect. Current animal studies should provide definitive answers to some of the questions about dose rate effects.

Because of the increasing costs of animal studies, we have designed an experiment that should allow us to determine the initial slopes of the dose response curves for the induction of myeloid leukemia by protracted neutron- and gamma-ray-radiation. From these results, dose rate effects, RBE values, and the risk of myeloid leukemia at low doses of either neutrons or gamma rays will be determined. Chromosomal and molecular studies are being carried out on mice in the same experiment in order to investigate the role of a specific chromosome aberration and the changes at the gene level relevant to development of myeloid leukemia.

REGULATION OF GENE EXPRESSION

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The objectives of this research are to define in molecular terms the mechanisms controlling expression of specific genes in mammalian cells, how these mechanisms operate during differentiation, and how they are regulated by hormones and other specific effectors. Current focus is on a gene we have cloned and characterized that is transcriptionally enhanced by glucocorticoids and by each of the usually antagonistic hormonal agents, insulin and cyclic AMP. We have provisionally designated this as "gene 33" pending identification of the protein product(s). The response of this gene to insulin in cultured hepatoma cells is especially large and rapid, providing a model for analysis of the

mechanisms by which interaction of this hormone with its membrane-bound receptor is realized as enhanced transcription of a specific nuclear gene.

Hormonal Regulation

We have used several approaches toward defining sequence elements in the DNA within or flanking gene 33 that may be involved in its transcriptional enhancement by hormones, with emphasis on insulin as an almost totally undefined regulator of gene expression.

Recombinant plasmids were constructed in which gene 33 5'-flanking DNA sequences were placed upstream of the *E. coli* chloramphenicol acetyltransferase (CAT) gene; these were transfected into cultured cells via $CaPO_4$ precipitation and the transcriptional promoting capacity of the gene 33 DNA determined as transient expression of CAT activity. Constructs containing 0.5 or 2.6 kbp of this rat DNA were very effective in driving CAT expression, and this expression was significantly enhanced by insulin. However, in these transient expression assays the insulin effect was not sufficiently large to support further experimentation.

Stable transformants of rat hepatoma cells were derived by cotransfection with gene 33-CAT constructs and another plasmid conferring neomycin resistance, followed by isolation of cell lines expressing significant levels of the bacterial enzyme. Several such lines have been developed wherein CAT expression is driven by 0.5 or 2.6 kbp of the 5'-flanking DNA of gene 33. Basal expression from the larger construct was consistently severalfold higher than from that containing only 0.5 kbp of rat DNA, suggesting the presence of strong basal enhancer elements in the upstream DNA. Physiological concentrations of insulin increased CAT expression six- to ten-fold in cells containing the smaller construct and somewhat less in those with the larger one, establishing firmly that insulin-responsive sequences reside in the 507 bp of rat DNA (-480 to +27) flanking the start site of gene 33 transcription.

To define further the specific sequence(s) required for response to insulin, we have used restriction enzyme and exonuclease III digestions to derive a series of CAT-expressing plasmids driven by truncated or otherwise deleted versions of the gene 33 DNA. Since the derivation of stable transformants is both time-consuming and costly, we have turned to transcription in cell-free extracts as an expeditious means of assaying promoter activity of these constructs. Results to date are very promising, in that extracts of nuclei from insulin-treated cells produce CAT mRNA at a rate 5 to 8 times greater than do control extracts, when the original construct containing 507 bp of rat DNA is used as template. Preliminary analyses of truncated constructs indicate that

insulin-responsiveness is retained when all but 100 bp of 5'-flanking DNA is removed. If confirmed this result will place the insulin-responsive sequences directly within the promoter region of gene 33, as this 100 bp contains basal transcription elements including a CAAT box (-89), SP-1 binding site (-67) and TATA box (-29). Inducible enhancer elements within the promoter region are not very common except in genes implicated in growth control and regulated by agents such as cyclic AMP or tumor promoters.

Structure and Function of Gene 33 Products

We have established that this single copy gene encodes two discrete mRNAs (derived by alternative splicing) which potentially are translated into two discrete proteins of molecular mass 50 kDa and 43 kDa. An S1 nuclease protection assay has shown that in liver and kidney the smaller mRNA constitutes 5 to 10% of the total gene 33 mRNA; this assay will soon be extended to other tissues and the possibility of developmental or hormonal regulation of production of the alternative mRNAs explored.

To ascertain the physiological role of the putative protein products is among our major goals but has proven to be most elusive. The derived amino acid sequences are remarkably uninformative when scanned for potential functional elements and exhibit no significant homology to those in national data banks. However, from our work and that of other investigators with whom we have shared our cloned gene 33 DNAs, strong indications are emerging suggesting a role in growth control. These include: the gene is expressed and hormonally-inducible in all tissues examined; expression in cultured cells is serum-dependent and strongly enhanced by phorbol esters as well as by insulin, glucocorticoids and cycloheximide; expression in liver is specifically increased during the recovery phase after treatment with the hepatotoxin, CCl_4; the 5'-noncoding region of the mRNAs is unusual and resembles that found only in oncogene or other growth-related gene products.

As a direct approach to detection, assay, and characterization of the protein products, a 12 amino acid peptide was synthesized corresponding to residues 360-371 of the sequence, a region common to both isoforms. Antipeptide antibodies have been developed in rabbits and Western blots used in initial attempts to detect the putative proteins in extracts of tissues and cultured cells. A number of reactive protein bands are detected but as yet insulin- or glucocorticoid-inducible bands of 50 and 43 kDa are not apparent. A variety of procedures are being explored in attempts to resolve this, including preparation of a second, presumably more specific peptide from a highly unusual region of the sequence.

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INTRACELLULAR TURNOVER OF GENE PRODUCTS

K.-L. Lee D. M. Yang

Intracellular degradation of mRNA and protein products of genes is highly significant in determining cell phenotype but mechanisms of the degradative processes are poorly understood. Particularly obscure are the features determining why some mRNAs and proteins undergo turnover at rates as much as 100 times greater than average. Products of such "rapid turnover" genes are present in cells in vanishingly small quantities, making it impossible to detect intermediates generated in the course of their degradation. As a potential solution to this problem gene amplification techniques have been used to generate cell lines containing many copies of a synthetic "rapid turnover" gene. These cells produce relatively massive amounts of the mRNA and protein products of the artificial gene, which should facilitate precise analysis of their degradative intermediates. In this initial work emphasis was placed on mRNA turnover.

An expression vector containing a tyrosine aminotransferase (TAT) minigene, made up of both cDNA and genomic DNA components in order to ensure precise initiation and termination of transcription, was transfected into CHO cells together with another expressing the amplifiable gene encoding dihydrofolate reductase (DHFR). TAT is normally expressed only in liver, wherein both the mRNA and protein undergo rapid turnover ($t_{½}$ about 1.5 h). Cells resistant to increasingly high concentrations of methotrexate (DHFR amplification) were assayed for TAT activity and lines developed which express high levels of the enzyme (TAT coamplification). Western blots have established that the enzyme subunit size is correct (50 kDa) and Northern blots show the expected 2.4 kb band for TAT mRNA.

One CHO line expressing high levels of TAT activity was examined and found to contain a steady-state level of TAT mRNA that is roughly 1000 times

greater than that of rat liver or hepatoma cells. When transcription was blocked with actinomycin, the 2.4 kb mRNA was degraded with a half-life of 100 min, a rate virtually identical to that found in liver. Earlier experimentation had suggested that there may be liver-specific factors required for the rapid turnover of TAT mRNA; this finding excludes that possibility. Further, Northern blots exhibit a reproducible band of about 1.0 kb that appears to arise in the course of mRNA degradation. These initial results suggest that this may indeed be a feasible approach to the analysis of intracellular mRNA turnover.

A puzzling, but potentially important aspect of these analyses has developed from measurements of the protein product of the transfected minigene. When assayed immunologically, TAT protein is present at levels comparable to those of the mRNA, but enzyme activity is only about 10% of that expected. The possibility of a coding error in the synthetic minigene cannot yet be excluded but seems most unlikely from several considerations. It may be then, that cells not normally expressing this enzyme lack a factor required for proper folding of the polypeptide product of translation, or perhaps for subunit interaction to form the enzymically active dimer. These possibilities are currently being investigated.

MOLECULAR GENETICS OF CANCER

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To understand the molecular genetic mechanisms of carcinogenesis, we have continued to work on experimental models of the laboratory mouse and place major emphasis on retroviral and retrotransposable gene elements integrated in the germlines. Our working hypothesis is that the genomic stress caused by environmental carcinogenic agents, as well as the metabolic changes associated with the neoplastic process, may activate the mobile genes in the cell genome that carry both the insertion and the regulatory gene elements and thus are capable of inducing genomic instability and oncogene expression. Previous studies in this laboratory have led to molecular cloning and sequence characterization of three distinct classes of proviral genes in the mouse genome: first, the proviruses of murine leukemia viruses (MuLV); second, the "polytropic" and the "modified polytropic" families; and third, the MRL or MuRRs that appear to show tissue-specific expression, respectively, in the lympho-hematopoietic system, in liver and kidney, and in mature reproductive organs. In the past year,

work has been aimed at (1) elucidation of the possible functional significance of these proviral genes in the mouse genome, including characterization of regulatory sequence-binding nuclear proteins; (2) elucidation of nuclease hypersensitivity in selected proviral genes in nuclei of various mouse tissues; (3) computer analysis of nucleotide sequence data for predicting variable gene regions in the retroviral genome structure; (4) development of transgenic mice for characterization of tissue-specific regulatory elements in environmental carcinogenesis; and (5) preparation of oncogene-carrying retroviral vectors for studying co-carcinogenesis in the animal. Also, research has been initiated on the isolation of retroviral genes from human cancer cells.

Transgenic Mouse Approach

Both the microinjection and the animal facilities for developing the transgenic mouse have been established in our laboratory. The FVB/N strain, an inbred NIH Swiss mouse carrying FV-1^b locus, has been noted to produce fertilized eggs with pronounced pronuclei with few cytoplasmic granules (information provided by R. Woychik) and hence is a very suitable recipient strain. Pseudopregnant $B_6C_3F_1$ female mice are used as the surrogate.

We are using transgenic mice to investigate regulatory gene function rather than the coding sequence of a gene. For this purpose, the following seven recombinant DNA clones have been selected for microinjection and development of transgenic mouse lines.

(1) **p26L-CAT**: This molecular construct contains the long-terminal repeat (LTR) sequence of a male-specific provirus-like gene clone as the regulatory gene component and the chloramphenicol acetyl transferase (CAT) gene, as the structural (marker) gene component. Since the male-specific retroviral gene is expressed predominantly in the testis, the transgenic mouse model is useful for studying regulation of transposable gene elements in the male germ cells.

(2) **pL2-CAT**: A characteristic sequence component of the male-specific retrovirus-like element, called A-2 (located at the junction of reverse transcriptase and integrase gene sequence regions), is also found in a few autosomal MRL retroviral genes. The LTR of such a molecular clone, pL2, is linked to the CAT gene and will be used to investigate its specificity of expression and gene transposition in both the male and the female germ cells.

(3) **pBOR-Ha-v-ras**: This molecular construct carries an oncogene, Harvey v-ras, in the proviral structure frame of an endogenous ecotropic MuLV WN1802B clone, pWNB-5, that comprises the two LTRs, the primer-binding site, the packaging signal sequence and a small 5' portion of the *gag* gene. Thus, this

transgene should not only show tissue-specificity for lymphoid and hematopoietic organs in the expression of the inserted Ha-v-ras gene, but should also serve as a substrate for detecting retrotransposition in these tissues of the animal.

(4) **pBOR-IL3**: This molecular clone carries the complete interleukin-3 mouse cDNA sequence in the retroviral vector frame derived from ecotropic MuLV proviral clone pWNB5, and should be useful for studying the possible role played by interleukin-3 expressed in radiation-induced myeloid leukemogenesis.

(5) **pBOR-GMCSF**: The sequences in this molecular construct are similar to those of pBOR-IL3 except it contains the mouse GM-CSF cDNA and hence should be useful for analyzing the possible role played by this hematopoietic growth and differentiation factor in radiation leukemogenesis.

(6) **p16-Ha-v-ras**: The LTR of pRFM16, a polytropic MuLV-related proviral clone, presumably contains transcriptional regulatory elements for expression in the liver and the kidney. We have therefore constructed the recombinant clone p16-Ha-v-ras that carries the pRFM16 LTR and Harvey viral *ras* oncogene. Transgenic mice harboring p16-Ha-v-ras should be useful for investigating the possible effect of hepatocarcinogens.

(7) **pSVN-Bendo**: This molecular construct carries the MuLV integrase regulated by MuLV LTR and neomycin-resistance gene regulated by SV40 origin.

Several founder mice have been obtained for each of the seven recombinant DNA constructs. (About 20 to 30% of the live baby mice developed from the microinjected and transplanted embryos were found to be positive.) Breeding programs are being performed to segregate individual transgenes and also to obtain homozygosity. Assessment of tissue-specific expression by *in situ* hybridization analysis is in progress for the p26L-CAT and the pL2-CAT transgenic mouse lines. Cell cultures from the tail and F1 embryos from male founder mice are also carried out to study the functional expression of transgenes *in vitro*.

Retroviral Vectors for Co-carcinogenesis Studies

Neoplastic transformation by oncogenes has been studied mainly in cultured cells *in vitro*, while most of the *in vivo* studies with viral oncogenes (e.g. promotion of skin carcinogenesis by Harvey viral *ras* oncogene) have been carried out with viral preparations containing the replication-competent "helper" viruses. We have therefore developed a few oncogene-carrying retroviral vector systems for the purpose of examining the possible role played by individual altered oncogenes in the multifactorial carcinogenesis process *in vivo*.

The oncogene components we selected for construction into the retroviral vector frame (i.e., LTR-pbs-psi-Δ gag-X-LTR, X being the oncogene insert) include Kirsten v-ras, Harvey v-ras, v-raf, v-fos, cDNAs of interleukin-3 and GM-CSF, and integrase coding sequence of Gross N-tropic and WN1802 B-tropic murine leukemia viruses. For retroviral packaging, we have employed PA317 and Ψ2 cell lines as well as ecotropic and amphotropic packaging cell lines that we have developed from mink CCL64 cells. Following the initial DNA transfection procedure, a "ping-pong" technique was employed to amplify the virus titer to about 10^6 focus forming units per ml.

In preliminary studies, up to 10^6 units of retroviral vectors carrying Ki-v-ras or v-raf were inoculated by intravenous or intraperitoneal injections into two groups of 6- to 16-week-old C_3H or $B_6C_3F_1$ mice, one receiving sublethal dose of CCl_4 1 day before virus inoculation and the other receiving the corn-oil solvent. In these strains of laboratory mice, combined treatment of Ki-v-ras and CCl_4 induced a high incidence of hepatocellular carcinomas beginning at 4 months following the treatment, while v-raf in combination with CCl_4 was much less effective. Mice receiving combined treatment of oncogene-carrying retroviral vectors and corn-oil or mice receiving CCl_4 treatment without subsequent viral inoculation showed no hepatoma development up to 8 months of observation. When similar experiments were performed with the RFM/Un strain mice, no hepatocellular carcinomas were obtained, regardless of whether or not combined treatment of CCl_4 and the Ki-v-ras-containing virus was given. However, inoculation of the Ki-v-ras virus either by itself or with CCl_4 was found to cause lymphomas in RFM/Un mice. These preliminary results indicate that the induction of hepatocellular carcinomas by Ki-v-ras oncogene is dependent on prior hepatocellular damage or regenerative changes from CCl_4 exposure and occurs only in the susceptible C_3H and $B_6C_3F_1$ laboratory mice. The long latency period between the retroviral oncogene inoculation and the emergence of hepatoma suggests that other genetic factor(s) are also involved in hepatocarcinogenesis.

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MONOCLONAL ANTIBODIES FOR DIAGNOSIS AND THERAPY

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Basic Carcinogenesis

Cell surface proteins mediate interaction between cells and their environment. In addition to uptake of nutrients, cells receive specific stimuli from growth factor-receptor interaction at the cell surface and specific attachment to substrate. The expression of these molecules can be altered in tumor cells and the alteration may affect growth characteristics of the cells.

A tumor surface protein of 180,000 Mr (TSP-180) has been identified on cells of several lung and mammary carcinomas of BALB/c mice. TSP-180 was not detected on normal lung tissue, embryonic tissue, or reticulum cell or other sarcomas, but it was found on lung carcinomas and on a melanoma from other strains of mice. Considerable amino acid sequence homology exists among TSP-180s from several cell sources, indicating that TSP-180 synthesis is directed by normal cellular gene(s) although the protein is not expressed at high levels in normal cells. TSP-180 has recently been identified as the $\alpha^6\beta_4$ integrin heterodimer. Even though integrins are involved in attachment to extracellular matrix, no function has been identified for $\alpha^6\beta_4$.

Alpha$^6\beta_4$ molecules have been identified on four different human carcinomas (lung, breast, embryonal, and colon). Evidence indicates that the human proteins are analogous to, but distinct from, those on mouse cells. Two-site monoclonal antibody (MoAb) assays for $\alpha^6\beta_4$ in the mouse and the human have been developed which allow quantitation of low levels (ng/mg protein) of these proteins in normal and neoplastic tissue. The results of

experiments in the mouse indicate that tumors have 10-100 times more $\alpha^6\beta_4$ than do normal tissues. In normal tissues, trace amounts of $\alpha^6\beta_4$ can be detected in lung and leg muscle, but not in heart, liver, kidney or spleen. Benign adenomas have small amounts of this integrin and the amounts increase as the adenomas become larger or progress to adenocarcinomas. All murine lung carcinomas tested to date (including primary, spontaneous, or chemically induced from different strains of mice) contain high levels of $\alpha^6\beta_4$ as do BALB/c mammary carcinomas.

Analyses of human tissue show that $\alpha^6\beta_4$ is expressed at low levels in nearly all normal organs but is elevated in some carcinomas, i.e., colon, lung, and larynx. In contrast, no detectable levels of this integrin were found in human breast carcinomas. Expression of β_4 protein is restricted to certain squamous epithelium, particularly of skin and colon.

Molecular characterization of the $\alpha^6\beta_4$ complex has revealed five distinct glycoproteins on SDS-PAGE analysis. The bands 1-3 (Mr 204, 185 and 150 kDa) are different forms of the β_4 molecule while bands 4 (Mr 135) and 5 (Mr 116) represent precursor and mature α^6, respectively. The band 1 protein of β_4 is phosphorylated in the C-terminal domain. Second generation MoAb to $\alpha^6\beta_4$ have allowed construction of 2-site assays specific for each of the protein bands. Expression in colon and lung tumors is restricted to band 3 β_4 protein.

Current work is focused on molecular characterization of $\alpha^6\beta_4$ and attempts to identify a specific receptor function for the complex. Molecular characterization is being approached by molecular cloning. A cDNA library of Line 1 cells in λgt11 has been screened with a rabbit antiserum to $\alpha^6\beta_4$ and a consensus oligonucleotide from the N-terminal sequence of band 5. Several phage isolates are being characterized which appear to contain fragments of $\alpha^6\beta_4$ specific cDNA. This cDNA will be used to analyze mRNA sizes and synthesis as well as the gene's size and position in normal and neoplastic cells. The effects of known factors on the phosphorylation of β_4 and growth of Line 1 cells are being analyzed to establish a function for this complex.

MoAb to normal mouse lung cells have been developed to study the role of normal cells as precursors of different types of lung cancer and to probe the interaction among cells during metastasis to the lung. Rat MoAb to mouse lung macrophages, Type I alveolar cells and endothelial cells have been identified and characterized. The most remarkable of these are two antibodies that recognize different epitopes on a 112-kDa glycoprotein (P112) expressed exclusively on lung endothelial cells. This is the first demonstration that endothelial cells in the lung are different from those in other organs in that they express a unique surface glycoprotein. The MoAb to this protein may be useful for organ specific

drug delivery, for studying the interaction between tumor cells and endothelial cells, and for "negative imaging" of tumors for diagnosis. Recent studies with liposomes targeted with these MoAb have shown for the first time that liposomes can be localized efficiently *in vivo*. Immunohistochemical staining of sections of mouse embryos indicates that P112 may play a role in embryogenesis. An alternative function as a lymphocyte homing receptor is also being explored.

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INDUCTION AND PROGRESSION OF CARCINOGENESIS IN THE RESPIRATORY TRACT

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A clear understanding of how potential carcinogenic agents, including chemical and radioactive byproducts of the nuclear energy cycle may cause lung cancer requires analysis of several steps in the carcinogenic process. It is necessary to (a) study the initial interaction of one or more of the hazardous agents with macromolecular components of the target cells; (b) determine whether the agent(s) induce cancer or promote the response of initiated cells; and (c) identify the molecular and cellular changes that occur in the target cells during the multiple stages of carcinogenesis. To investigate these aspects of respiratory tract carcinogenesis, we have developed *in vivo-in vitro* and *in vitro* rat tracheal cell models; and some recent studies using these models are discussed below.

Promotion, Co-carcinogenesis, and Dose Effects in *In Vivo-In Vitro* Tracheal Implant Model

In vivo models of tumorigenesis are essentially limited to morphological endpoints. To overcome this limitation a combined *in vivo-in vitro* model of carcinogenesis, the tracheal implant, was developed several years ago. In this model, rat tracheas are implanted on the backs of isogenic hosts to serve as a well-defined target organ for exposure. The sites of tumor initiation (TIS) are detected as carcinogen-altered cells selected by placing primary cultures established from pieces of the tracheal implants in a medium deprived of pyruvate, a component we found to be necessary for the long-term growth of normal tracheal epithelial cells, but not for altered cells. Our early studies demonstrated a direct correlation between extent of exposure to the carcinogen, dimethylbenzanthracene (DMBA), and number of TIS isolated from the tracheas, as well as expression of the other markers indicative of neoplastic progression in these cell populations, such as anchorage-independent growth in soft agarose and the continuation of nuclear division in the presence of cytochalasin B. Also, since lesions on the explants are initially identified from cytopathology of

exfoliated cells, cellular and biochemical properties of cell populations derived from the specific lesions were studied and were found to correlate directly with conventional morphological markers of the progression of neoplasia.

Recently, we modified the tracheal implant model into an open-ended system. In the open-ended tracheal implant model, this well-defined target site can be exposed an unlimited number of times to single- or multiple-test agents of any physical form. With this approach we have studied the relative potency of DMBA, benzo(*a*)pyrene (B(*a*)P), and formaldehyde (HCHO) to initiate carcinogenesis in the tracheas. Assuming linear relationships between carcinogen dose and induction of tumor-initiation sites, DMBA was 36 times more potent than B(*a*)P, and HCHO was a very weak carcinogen. However, when HCHO was given as a promoter following B(*a*)P initiation, a fivefold increase in TIS was obtained above that found with B(*a*)P alone. HCHO also acted as a strong co-carcinogen if repeatedly given 24 h after B(*a*)P in a twice weekly exposure regimen for 4.5 months. These results have far-reaching implications if extrapolated to the human situation, for they suggest that humans that have been exposed to polycyclic hydrocarbons may be far more susceptible to tumor development if exposed to HCHO. The findings are also consistent with the high incidence of bronchial carcinomas found in tobacco smokers, since tobacco smoke contains considerable amounts of both polycyclic hydrocarbons and HCHO. Currently, we are investigating the influence of a second late exposure to carcinogen on the progression of carcinogenesis in the tracheal implants.

Changes in Growth Regulation and Oncogene Expression during Respiratory Carcinogenesis

A major interest in our laboratory is to identify key biochemical and genetic changes in carcinogen-exposed tracheal cells and to determine how they relate to the evolving changes in growth regulation found during the progression of neoplasia. Since we found that the ability of tracheal cells to survive in pyruvate-deprived medium is a very early marker of carcinogen-induced alterations, we have attempted to elucidate the cellular mechanisms underlying the requirement of pyruvate by normal tracheal cells in culture and the lack of this need by the carcinogen-altered cells. Two key earlier findings are: (1) normal tracheal cells actually metabolize [^{14}C]pyruvate for macromolecular synthesis and lactic acid production at 3-4 higher levels than carcinogen-altered cells; (2) carcinogen-altered cells and tumor cells have markedly higher levels of particulate-bound $NADP^+$-dependent malic enzyme activity. This enzyme catalyzes the formation of pyruvate from malate. Recently, we have established conditions for culturing dissociated normal tracheal epithelial cells in a 1% serum-supplemented or a serum-free medium and have obtained preliminary evidence that the pyruvate may be converted in some way or bound to an amino acid before it can be

utilized by these cells to support cell proliferation. In NMR spectroscopy studies carried out in collaboration with M. Buchanan of the Analytical Chemistry Division, we have tentatively identified a pyruvate-amino acid complex in our culture medium. Collaborative experiments aimed at further characterization of this complex, as well as identification of other metabolites and their differences in normal and carcinogen-altered cells from different stages of carcinogenesis, will continue.

Our *in vivo-in vitro* experiments have generated many cell populations, with well-defined growth properties representing several stages in the progression of neoplasia, that are particularly amenable for analysis of changes in gene expression at these times. In collaboration with G. N. Cosma and S. J. Garte of New York University, we are examining oncogene expression in these cell populations. We have found stage-specific progressive increases in C-*myc* gene expression. H-*ras* oncogene was expressed at all stages, and alterations at codon 61 were identified by restriction fragment-length-polymorphism (RFLP) analysis in some of the cell lines. These altered DNA fragments have been amplified by the polymerase chain reaction (PCR) technique for nucleotide sequence analysis to determine nucleotide transitions that may alter gene expression and ultimately growth control during carcinogenesis.

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RADIATION CARCINOGENESIS

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The radiation carcinogenesis program has the advantage of collaborative studies both within the division and with other laboratories. For example, collaborative studies are being carried out with E. Alpen, P. Risius-Powers, and S. B. Curtis at Lawrence Berkeley Laboratory, on heavy ion radiation carcinogenesis. These studies are concerned with the relationship of linear energy transfer and cancer induction. The experiments are also providing information about relative biological effectiveness of the various radiations encountered in space.

The collaboration with R. D. Ley, Lovelace Foundation, Albuquerque, on ultraviolet traduction (UVR) carcinogenesis continues and of late has concentrated on the mechanism of UVR-induced tumors in *Monodelphis domestica*, an opossum with photoreactivating enzymes. In this model system it is possible to answer questions about the role of pyrimidine dimer induction in UVR-induced biological effects.

A collaborative study is being carried out by M. Terzaghi-Howe with R. J. Preston of the Molecular Cytogenetics Section and W. K. Yang on the induction of neoplastic change in tracheal epithelial cells by alpha particles. These studies are part of DOE's radon program.

Influence of Age, Sex and Genotype in Radiation Carcinogenesis

A collaborative study has been carried out with B. A. Carnes, Argonne National Laboratory, on the influence of age, sex, and genotype on radiation carcinogenesis, the results of which have been published. Although age is a major determinant of cancer rates, susceptibility for cancer induction of a number of cancers decreases with age. The Weibull model was used to test the influence of age at exposure, of sex and of radiation quality using median lifetime for each of the selected groups of cancer. Age and sex were used as covariables. The influence of sex varied with the type of cancer. For example, lymphomas and lymphoblastic leukemias were more susceptible to induction in females than males, whereas males were more susceptible to the induction of lung tumors. We concluded from this study that any age-dependent difference in sensitivity for life shortening, caused by radiation-induced excess cancer,

occurs at ages younger than 110 days. The decrease in susceptibility with age for induction of cancer by radiation is an intriguing question in terms of mechanisms. It is not known whether the major change in susceptibility with age is in initiation or the expression of the initial events.

We are carrying out a study of age-dependency of the induction of skin cancer by soft X rays. HRS/J mice have been exposed to fractionation regimens of 25 kV X rays at 8- and 18-weeks of age. The preliminary results indicate a distinct decrease in susceptibility with age despite the fact that the ages at first exposure differ by only 10 weeks. The second part of this experiment is aimed at the determination of whether modulating expression eliminates the age-dependent differences. This study has already indicated a difference in susceptibility between our two stocks of hairless mice, HRS/J and SKH-hr-1. The former is more susceptible to X-ray induction of squamous cell carcinomas. This result is somewhat surprising because SKH-hr-1 mice are considerably more susceptible than HRS/J mice to the induction of skin cancer by UVR.

Studies of the Effects of Dose Rate and Radiation Quality

There are two major questions about the effects of ionizing radiation that are not being answered by the many and large studies of human populations: (1) Are low dose-rate exposures to low-LET radiation less effective than high dose-rate exposures and, if so, by how much? (2) How much greater are the carcinogenic effects of high-LET radiations (neutrons, alpha particles, and heavy ions) than low-LET radiations? Both these questions are being investigated in current experiments. Two experiments that are designed to study the dose-response relationship of the induction of cancer by neutrons are near completion. The results should establish whether fractionation or protraction increases the carcinogenic effects of neutrons. Furthermore, the results of the study of multiple exposures to small doses of neutrons will provide information required for modeling of the dose-response relationship of the induction of various types of tumors.

Radiation-Induction of Myeloid Leukemia

A specific chromosome translocation is considered to be causally related to naturally-occurring, chronic myeloid leukemia in humans. However, it has not been determined whether the induction of a specific chromosome aberration by carcinogenic agents, such as ionizing radiation, is causally related to the induction of myeloid leukemia. In mice, particularly in the RFM and CBA strains, a deletion in chromosome 2 is found in almost all naturally-occurring- and radiation-induced-myeloid leukemias. There are a number of genes of interest in the region of the breakpoints involved in the deletion, for example, c-abl, which

is considered to be the oncogene involved in chronic myeloid leukemia in humans.

In the current study, in collaboration with R. J. Preston, the effects of dose rate and radiation quality are being studied at the whole animal, tissue, and chromosomal level. The molecular studies are aimed, initially, at characterizing the structural and functional changes in the genes involved in the deletion. It is not yet clear whether the changes at the gene level are limited to chromosome 2. There is a marked difference between male and female RFM mice in both the incidence of naturally-occurring myeloid leukemia and in susceptibility to induction of this leukemia by radiation. It seems unlikely that a similar, sex-dependent susceptibility exists for the induction of the specific chromosome aberration that appears to be associated with myeloid leukemia. It is more likely that there is a lower probability of the essential post-initiation events in females than in males.

In solid cancers there is good evidence that while the initial events are a prerequisite for carcinogenesis, they do not determine the probability that a cancer develops. This fact makes it imperative to study the factors influencing the expression of initiation or neoplastic transformation. The studies carried out with tracheal epithelial cells are aimed at determining the mechanisms by which cells and tissues attempt to control the development of tumors.

Effect of Cell-Cell Interactions on Development and Expression of Transformation in X-Ray-, Neutron-, and Alpha-Particle-Exposed Rat Tracheal Epithelial Cells

A combined *in vivo-in vitro* model has been utilized to evaluate the influence of cell-cell interactions on expression of radiation-induced transformation in rat tracheal epithelial cells. Two types of interaction are evaluated. One appears to require the intact tissue structure with direct cell-cell contact. The second is observed in cell culture and appears to involve "communication" between cells by means of a diffusible factor in the culture medium. Our experiments indicate that some factor(s) is produced by normal cells in culture at a high cell density that is capable of influencing proliferation. When low density cultures of normal cells (~50 colonies per dish) are fed medium from high density cultures (~300 colonies per dish), called conditioned medium, the frequency of proliferating epithelial cell foci was reduced by an order of magnitude. The factor involved appears to be transforming growth factor type β (TGF-β).

It has been established that carcinogenic agents, such as radiation, initiate a number of cells that is greatly in excess of the number of tumors that develop. Therefore, it appears that expression of the initial events is suppressed. This suppression is a characteristic of certain tissues, in particular epithelial. We have

established that cell-cell interaction in intact tissue appears to moderate expression of preneoplastic- and neoplastic-alteration induced by low-LET radiation (X rays), but not those induced by high-LET radiation (neutrons and α-particles). Our results indicate that the environment in which cells are irradiated has a marked influence on the induction of cell populations that show the initial changes associated with neoplasia. However, the influence of environment was, in turn, dependent on radiation quality.

Modulation of Transforming Growth Factor Type-β (TGF-β)-Induced Inhibition of Growth by Cell-Cell Interactions

When isolated normal and preneoplastic tracheal epithelial cells are exposed to TGF-β in culture, inhibition of growth is observed. In the presence of 0.5 ng/ml TGF-β, both the uptake of initiated thymidine and the formation of colonies are reduced 100-fold below control levels. If, however, cells are exposed either within the intact tissue or as part of a colony in culture, no inhibition is observed. This effect appears to be dependent on direct cell-cell contact. Experiments are under way to gain some insight into this phenomenon.

Time Course of Cell-Cell Interaction-Induced Modulation of X-Ray-Induced Transformation in Rat Tracheal Cells

Cells are exposed within the intact tissue to 4.5 Gy X rays and then enzymatically harvested as a single cell suspension from the intact tissue 0-24 h after irradiation. With increasing times after exposure, cells left in the intact tissue exhibit a decline in transformation frequency. In preliminary experiments a return of transformation frequencies to control levels was observed 4-6 h after exposure.

Alpha-Particle-Induced Alterations in Exposed Rat Tracheal Epithelial Cells

We are currently carrying out experiments aimed at establishing baseline survival and transformation frequency data for rat tracheal epithelial cells exposed to alpha particles. Cells are exposed, either as a single cell suspension or as part of an intact tissue segment, directly on a ^{210}Po-platinum α-particle source.

The D_{37} for normal tracheal epithelial cells and for the preneoplastic cell line XR600 is observed when cells are exposed directly on a 9.6×10^6 dpm ^{210}Po source. This activity has been calculated to yield roughly 5 α-particles per cell, 3 α-particles per nucleus, and a dose of 1.5 Gy.

Maximum transformation frequency (1.1%) was observed when cells were exposed on a 2.5×10^6 dpm ^{210}Po α-source, roughly corresponding to 1 α-particle

per nucleus and a dose of 0.4 Gy. Further increases of dose were not associated with further increases in the frequency of transformation.

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Molecular and Cellular Sciences Section

Section Overview - J. S. Cook

The various projects in this Section are directed toward understanding at the most basic level the mechanisms of life processes that relate to the overall mission of the Biology Division. Programs are concerned with the structure of DNA and its organization in the eukaryotic genome, the structure of chromatin, DNA-repair processes, and enzyme mechanisms, all problems underlying the cellular functions and the genetic responses of organisms to environmental factors. Other programs of the Section, also relating organisms to their environment, are concerned with the mechanisms of transport of metabolites by mammalian cells and the responses of cells to freezing. The latter program also has an applied component in the preservation of valuable mutants. Some of the significant findings of the various groups are encapsulated here to demonstrate how the ongoing work is not only of interest as basic science but also relates to the programs of the DOE.

Together, the Protein Engineering Group and the Protein Chemistry Group form the largest unified cohort in the Section. They use the methods of site-directed mutagenesis, along with other sophisticated biochemical and genetic techniques, to explore structure/function relationships of several proteins of both plant and animal origin. Although the proteins form a diverse group, they are each of significance to various aspects of the mission of the Biology Division and include energy-related enzymes, growth factors important to cancer biology, and repair enzymes that function in the responses of cells to physical and chemical mutagenic agents. This group has also been developing vectors for cloning very large DNA fragments that could potentially accelerate 10- to 100-fold the mapping phase of the Human Genome Initiative.

The DNA Repair and Mutagenesis Group focuses on the mechanisms of mutation induction in human cells and repair of alkylated DNA by human repair enzymes. The group has successfully cloned the cDNAs for two human enzymes that repair alkylation damage. They are the first cDNAs to be cloned for human repair enzymes of known biochemical activity. The selection strategy is based on the repair of alkylation damage in repair-deficient *E. coli* and the consequent survival of these cells when the repair proteins encoded by the cDNAs are expressed in them.

The group studying the Structure and Organization of a Eukaryotic Genome has extended the fine analysis of the relation of DNA primary sequences in their model system (a complex satellite of a crustacean) to mutation hotspots,

showing that regions of sequence-dependent distortion of the helix are regions of high frequency mutation. The satellite sequence is transcribed in specific organs at specific growth stages, and the regulation of such transcription is under current investigation.

The group investigating the Structural Biology of Nucleosomes and Genes has analyzed in detail their 8 Å crystallographic solution of the nucleosome core particle structure and is planning for crystals that will allow a 3 Å solution. For this purpose they have reconstituted three preparations of precisely positioned nucleosomes that are each homogeneous in their histone composition and in the associated DNA sequence, and they have shown that the reconstitutions conform to theoretical predictions. This is one of two groups in the world to have succeeded in crystallizing nucleosomes. The DNA wrapping around the protein core of the nucleosome does not form a smoothly curved superhelix but at several locations makes sharp histone-induced bends related to the primary DNA sequence at those points. These are putative binding sites for DNA binding proteins. The analysis has the promise of establishing the structural basis for the regulation of transcription and other DNA-dependent functions of the cell.

Studies on the regulation of eukaryotic messenger RNA synthesis and turnover have focused on several enzymes important in mRNA metabolism, including protein kinases from HeLa cells that phosphorylate a terminal repeating sequence of the large subunit of RNA polymerase II, a decapping enzyme that removes the 5' terminal cap structure of mRNA and thereby renders it susceptible to degradation, and a 3' → 5' exoribonuclease that degrades decapped RNA. The interactions of the activities of these enzymes appear to play important roles in regulating mRNA concentration and translation. In addition, 5' → 3' exoribonuclease from yeast has been cloned and characterized. Another yeast enzyme, poly(A) polymerase that is responsible for the polyadenylation of mRNA, has been purified 1000-fold as a first step in the cloning of its gene.

The Membrane Biology Group has continued its studies on the regulation of transport systems in relation to cell growth and differentiation. Three major projects are concerned with (1) the role of protein kinase C in the regulation of amino acid transport, (2) coupled gene expression for hexose transporters in differentiating renal proximal tubule cells in culture, and (3) cellular responses, including transport modulation, to engineered hEGF variants provided by the Protein Engineering Group.

The Cryobiology Group has further supported its contention that an important, but frequently neglected component of freezing injury is the crowding of cells in unfrozen channels during ice formation. Collaborating with laboratories

cryobiology of human and bull sperm and applied studies in freezing of embryonic mouse and *Drosophila* for the cost-efficient, long-term banking of mutant stocks. This year they have achieved an important breakthrough in finding the means for permeabilizing *Drosophila* embryos to cryoprotectants and water while maintaining high viability.

Several members of the Section are collaborating with scientists in other divisions on problems in Structural Biology. These projects (and the other divisions concerned) include studies on DNA and other macromolecules by scanning tunneling microscopy (Health and Safety Research Division); sequencing by Fourier transform mass spectrometry of mutagenized DNA fragments, including the specific identification of alkylated isomers of altered bases (Analytical Chemistry Division); structural studies on cyclodextrins to explore hydrogen bonding by neutron scattering, and refinement of the nucleosome studies described above (Solid State Division). These projects form the nucleus of the Structural Biology Program.

PROTEIN ENGINEERING AND PROTEIN CHEMISTRY

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The unifying theme in this group is the use of site-directed mutagenesis and chemical approaches to elucidate structure/function correlations of proteins. Investigations focus on four proteins with quite diverse functions: ribulose bisphosphate carboxylase/oxygenase, an enzyme whose activity is a major determinant of biomass yield; phosphoribulokinase, a photosynthetic enzyme whose activity is regulated by light; epidermal growth factor, a hormone that regulates cellular growth and differentiation; and O^6-methylguanine-DNA methyltransferase, a protein that repairs alkylated DNA.

Ribulose Bisphosphate Carboxylase/Oxygenase (Rubisco)

Ubiquitous among photosynthetic organisms, Rubisco is essential for net conversion of atmospheric CO_2 into carbohydrates. Thus, this enzyme, a cornerstone of living processes, serves a critical role in the production of biomass for energy and is relevant to the global CO_2 issue (i.e., the greenhouse phenomenon). The enzyme is bifunctional: in addition to catalyzing the carboxylation of D-ribulose-1,5-bisphosphate to yield two molar equivalents of D-3-phosphoglycerate (the CO_2-fixation reaction), it also catalyzes the oxidation of ribulose bisphosphate by molecular oxygen to yield one molar equivalent each of phosphoglycolate and 3-phospho-glycerate. Although multiple substrate specificities among enzymes are not unusual, the bifunctionality of Rubisco is perhaps unprecedented in that the two reactions catalyzed are the initial steps in competing metabolic pathways -- photosynthetic assimilation of CO_2 and photorespiration, the latter an energy-wasteful process which results in the release of previously fixed CO_2.

Our goals are (1) to understand the mechanism of this complex enzyme, especially the precise catalytic roles of active-site residues, and (2) to evaluate the feasibility of improving the carboxylase/oxygenase activity ratio and thereby providing an approach to enhancing biomass yields.

In contrast to Rubisco from higher plants, which is comprised of two gene products (eight large and eight small subunits per molecule of enzyme), the functionally analogous enzyme from the purple, non-sulfur photosynthetic bacterium *Rhodospirillum rubrum* is a homodimer and the product of a single gene. This enzyme has thus been used for all of our mutagenesis studies, whereas chemical modifications have involved both types of the enzyme.

In the absence of 3-dimensional structural information but guided by results of affinity labeling and comparative sequence considerations, our laboratory has used site-directed mutagenesis to address a variety of structure-function issues: validation (or invalidation) of chemical modification data, location of the active site (i.e., intra- *vs*. intersubunit), subunit-subunit interactions, and function of active-site residues. Examination of the ability of mutant proteins, devoid of carboxylase activity, to catalyze discrete partial reactions in the overall process has provided a useful approach for defining the function of active-site residues. For example, replacement of Lys166 with glycine abolishes carboxylase activity, but the resulting mutant protein is still capable of catalyzing the conversion of the 6-carbon reaction intermediate to 3-phosphoglycerate (collaboration with G. H. Lorimer of DuPont). Replacement of Lys329 with glycine also abolishes carboxylase activity; however, in contrast to the Gly166 mutant protein, the Gly329 mutant protein is a catalyst for the enolization of ribulose

bisphosphate, the initial step in overall carboxylation. These observations suggest that Lys166 functions as the base that abstracts the C3 proton from ribulose bisphosphate and that Lys329 enhances the reactivity of the resultant enediol.

As a potential approach to altering the carboxylase/oxygenase activity ratio, we have used chemical modification in concert with mutagenesis to replace active-site lysines with aminoethylcysteine and to replace an active-site glutamic acid with carboxymethylcysteine, thereby introducing subtle changes into the active-site microenvironment.

The three-dimensional structure of Rubisco has been elucidated very recently by Carl Branden and colleagues at the Swedish University of Agricultural Sciences and by David Eisenberg and colleagues at UCLA. The crystallographic data confirm the active-site location and the identities of several active-site residues as deduced from chemical and mutagenesis studies but raise questions about precise roles of these residues that must be resolved.

Vector Development

Alteration of proteins by site-directed mutagenesis requires extensive use of recombinant DNA manipulations and exploitation of plasmid and phage biology. As part of our efforts to modify Rubisco, we have developed a pair of plasmids that integrate and simplify the construction, characterization, and expression of mutant genes. These matched plasmids draw together many features of various cloning and expression vectors, which expedite the preparation of site-directed mutants, from the mutagenic oligonucleotide to the expressed product. This matched pair of vectors merge many advantages: high inducible levels of the desired gene product, accomplished without forming a NH_2-terminal fusion-protein; facile production of single-stranded DNA template for mutagenesis and sequencing; stable, compatible plasmid replication origins for the formation of hybrid enzyme through *in vivo* complementation, or the coincident production of two different recombinant proteins. One plasmid contains the ColE1 replicon while the second has the p15A replicon. Thus, the two plasmids can be stably coexpressed in a common host. This allows the facile construction of hybrid proteins *in vivo*. Both plasmids contain the f1 IG region, and thus in the presence of helper phage, single stranded DNA is produced. This is necessary for site-directed mutagenesis and for DNA sequencing. Both plasmids allow the production of native protein from the tightly regulated *tac* promoter and *lac* operator. The desired proteins can then be expressed at high yields. Recent experience with the *rbc* gene in these new vectors has shown that new mutants can be synthesized and characterized in six to ten working days, with enzyme yields of 5-10% of cell protein.

Phosphoribulokinase (PRK)

PRK catalyses the ATP-dependent synthesis of ribulose bisphosphate in the illuminated chloroplast stroma. The enzyme is activated by thioredoxin-mediated reduction of a disulfide, which we have shown to be intrasubunit and comprised of Cys16 and Cys55. Our chemical modification studies revealed that Cys16 is located within the ATP-binding domain of the active site but is not required for catalysis. The hyper-reactivity of Cys16 has complicated the identification of other active-site residues; however, Cys16 may be selectively methylated with retention of 50% of the original kinase activity. Potential active-site reagents are now being screened against methylated PRK, in which Cys16 is no longer a target for modification.

The gene for the mature form of spinach PRK has been expressed in *Escherichia coli* as a prerequisite to site-directed mutagenesis. This was accomplished by restructuring the DNA in the PRK leader to introduce both a restriction endonuclease site, which was used to move the gene into an expression vector, and a properly placed initiation codon. We have observed that the mature spinach PRK has limited stability in *E. coli,* so it has been necessary to grow the bacteria under conditions which minimize heat shock proteases in order to maximize PRK production. We have also observed that by inclusion of arabinose in the culture, which presumably increases the intracellular pool of ribulose-5-P, the enzyme is further stabilized.

Epidermal Growth Factor (EGF)

Background. Because of their crucial role in the regulation of growth and differentiation and the possible abnormalities caused by their malfunction, there is considerable interest in the study of growth factors. Among the most highly studied growth factors is EGF, a 6-kDa protein (53 amino acids) with 3 internal disulfide bonds. EGF initiates its action by high-affinity binding to its specific receptor on the cell surface -- the EGF receptor. The receptor is a single polypeptide having an internal (cytoplasmic) domain exhibiting protein tyrosine kinase activity under the regulatory control of an external domain that binds the growth factor.

The binding of EGF to its receptor unleashes a cascade of biochemical reactions. These include stimulation of the receptor tyrosine kinase activity leading to autophosphorylation and phosphorylation of endogenous proteins, increased glycolysis and protein synthesis, and enhanced transcription of specific genes. This ultimately leads to increased DNA replication and cell division. Evaluation of the biological properties of the receptor has shown that the

receptor tyrosine kinase activity is essential for most of the events that lead to cell division.

Research goals and approach. One of the specific aims of our research project is to elucidate structure/function correlations of human EGF by the application of protein engineering. Utilizing the human EGF gene, recently cloned in this laboratory as a secretory protein in *Escherichia coli* (Engler et al., 1988), targeted amino acid residues in the EGF molecule were substituted by oligonucleotide-directed mutagenesis. The selection of amino acid residues for mutagenesis was based on (a) their interspecies homologies; (b) reported studies with chemically synthesized peptide fragments of EGF; (c) published solution structures of mouse and human EGF, which are based on 2-D NMR and computer modeling; and (d) our previous studies of site-directed mutagenesis of human EGF. Each mutant EGF was purified to homogeneity and tested as follows: (1) specific binding to the EGF receptor purified from human cells; (2) stimulation of the protein-tyrosine kinase activity of the EGF receptor; and (3) stimulation of DNA synthesis and growth of animal cells in culture.

Another goal of the research is the development of EGF analogs which, even upon saturation binding to the EGF receptor, are unable to elicit the full tyrosine kinase stimulation necessary for cell proliferation. Such analogs could be useful as inhibitors of cell growth and might possess anti-tumor activity.

Analysis of results:

1. The acidic residues, Glu24 and Asp27, in the B-loop, and the highly conserved Pro7 in the A-loop, do not seem to be important in determining EGF activity.

2. Aromatic residues -- Tyr13 in the A-loop, Tyr22 and Tyr29 in the B-loop, and Tyr37 in the C-loop -- are important in determining EGF's affinity to its receptor. It has been postulated, based on NMR studies, that aromatic side chains in the EGF molecule are in close proximity to one another. This thermodynamically favored structure may contribute to the folding and stability of EGF in its functional tertiary structure. Our mutagenesis experiments support the above and open up possibilities for the design of EGF analogs with either antagonist or increased agonist function.

3. Gly36, Gly39, and Arg41 in the C-loop (which are completely conserved in EGFs from different species, and related growth factors) are quite important in EGF's interaction with its receptor. Mutations at the two glycine sites appear to decrease the receptor binding affinity due to possible altered conformations of the mutant analogs. Studies with several mutations at position 41, on the

other hand, strongly suggest that the role of Arg41 is to serve as a contact point in EGF's interaction with its receptor.

4. Hydrophobic amino acids -- Val19, Met21, Ile23, and Leu26 in the B-loop, and Leu47 in the COOH-terminal tail -- are quite important in EGF's biochemical activity. It appears that Leu47 may also serve as a contact point in the interaction of EGF with its receptor. Ile23, Leu26, and Leu47 are particularly interesting because certain mutants at these sites, even upon saturation binding to the EGF receptor, are unable to elicit the full tyrosine kinase stimulation. Moreover, these mutants are able to displace wild-type EGF and inhibit the latter's stimulation of the receptor's tyrosine kinase activity. Thus, these mutants appear promising as possible growth inhibitors.

Conclusions. It is clear that amino acid substitutions by protein engineering have provided crucial information about the degree and nature of importance of specific residues in EGF. Certain mutants appear promising as anti-EGF and therefore antigrowth substances, and could be useful for potential cancer therapy. Such mutants are currently being tested in mammalian cell culture experiments for their effect on cell growth (see article in this volume "Regulation of Mammalian-Cell Transport Systems" by Cook et al.).

O^6-Methylguanine-DNA Methyltransferase (MGMT)

MGMT is a ubiquitous repair protein, which is specific for removal of the procarcinogenic and promutagenic DNA adduct, O^6-alkylguanine, induced by simple alkylating agents. This unique protein acts in a suicide reaction in which the alkyl-group is transferred directly from DNA to the protein in a stoichiometric fashion. The alkyl-acceptor residue in MGMT in all known cases is cysteine. The protein is highly regulated in both bacteria and mammals, and the elucidation of the basis of this regulation is crucial to the understanding of somatic and germ line mutations in mammals as well as the development of resistance of certain tumors to alkylating drugs.

In the last report, we described large-scale purification and characterization of the major MGMT of *E. coli*, namely Ada protein, encoded by a high expression recombinant plasmid constructed in our laboratory. With our recent success in cloning the cDNA of the human MGMT and expressing it in *E. coli* (see p. 15), we compared the primary sequence of the MGMTs. It is remarkable that while the overall sequence similarity between the two MGMTs (Ada and Ogt) of *E. coli* and the human protein is less than 10%, the sequences in the neighborhood of alkyl acceptor cysteine were similar to the extent of 60-65% (35-40 residues are either identical or similar in a stretch of 61 amino acids).

The human protein (22 kDa), comparable in size to the Ogt protein (18 kDa) and the C-terminal half of the Ada protein (19 kDa), is distinct in biochemical properties in that it accepts the alkyl group exclusively from O^6-alkylguanine, while O^4-alkylthymine is also a substrate for the proteins of *E. coli*. Furthermore, the human protein reacts at a rate a thousand-fold less than the Ada protein. Even though attempts to solve the crystal structure of Ada protein by G. Bunick's group in this Division have not yet been successful, we should be able to make certain reasonable changes in the sequence of human protein. In the first series of experiments, we plan to induce mutations in the human MGMT such that the active site region would be identical to that of the Ada protein. In the meantime, we are engineering our expression plasmid in order to increase the yield of human MGMT.

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GENETIC CONTROL OF MUTAGENESIS AND DNA REPAIR

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The *Saccharomyces cerevisiae* REV1 gene product functions in a cellular process required for mutagenesis caused by ultraviolet (UV) radiation and many chemical mutagens. The *REV1* gene is one of a small group of yeast genes, including *REV3*, *REV7*, *CDC7*, and *NGM2*, whose mutant alleles have a severe and general effect on mutability. Mutants of *REV1* exhibit little or no mutability either by UV light or by many chemicals, and are only slightly sensitive to the lethal effects of these agents.

The REV1 gene product is thought to compose part of a DNA-repair process that is "error-prone" or "mutagenic" in its function. Just as SOS processing by *umuDC* is distinguished from other Rec functions and from *uvrABC*-controlled excision repair in *Escherichia coli*, mutagenic repair is distinct from the other major repair pathways in yeast. Excision repair is controlled by genes of the *RAD3* epistasis group, and recombinational strand-break repair is controlled by genes of the *RAD52* epistasis group. The *REV1* gene is part of the *RAD6* epistasis group; the *RAD6* gene product has been identified as a ubiquitin transferase that may be required to process histones on chromatin to initiate DNA repair.

As an initial step toward understanding the regulation and function of mutagenic repair, we sought to clone and characterize the *REV1* gene. We hope to use such a clone to overproduce the REV1 gene product to facilitate purification, characterization, and *in vitro* reconstruction of the mutagenic repair complex. In this regard, other investigators have reported the sequence of the *REV3* gene, which appears to encode a DNA polymerase; a fragment which complements *rev2* has been identified; and *CDC7*, which potentiates mutability when present at high copy number, has been shown to encode a protein homologous to protein kinases.

We have determined the sequence of a 3,485 base-pair segment of DNA that complements the *rev1-1* mutant. Gene disruption was used to confirm that this DNA contained the *REV1* gene. The sequenced segment contains a single long open reading frame (ORF), which can encode a polypeptide of 985 amino acid residues. The *REV1* transcript is 3.1 kbp in length. Frameshift mutations introduced into the ORF yield a Rev$^-$ phenotype. A base substitution, encoding Gly193 to Arg193, is found in this ORF in *rev1-1*. Deletion mutants, lacking segments of the 5' region of *REV1*, have intermediate mutability relative to *REV1* and *rev1-1*; a complete deletion exhibits lower mutability than *rev1-1*. *REV1* is not an essential gene. An in-frame fusion of the 5' end of the *REV1* ORF to the *lacZ* gene produces β-galactosidase activity constitutively. The predicted REV1 protein is hydrophilic, with a predicted pI of 9.82. No homologies to RAD1, RAD2, RAD3, RAD7, or RAD10 proteins were noted. A 152 residue internal segment displayed 25% identity with UMUC protein. Both *REV1* and *umuC* are required for error-prone repair, functionally supporting the suggestion that these peptide segments are homologous. This alignment should prove useful as a basis for targeting mutations to identify functionally important segments of the REV1 protein.

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DEVELOPMENT OF VECTORS FOR CLONING LARGE DNA FRAGMENTS

F. W. Larimer R. J. Mural

The primary goal of this project is to explore techniques and develop vectors for cloning and maintaining large (100+ kbp) fragments of DNA. Vector systems with capacities in this range could accelerate the mapping phase of the Human Genome Initiative by a factor of 10- to 100-fold. The specific goals are:

1. To develop vectors for maintaining large segments of DNA in *Escherichia coli* using the plasmid prophage replicon of bacteriophage P1.
2. To optimize the introduction of such large constructs into bacteria.
3. To explore the use of site-specific recombination to facilitate the *in vitro* circularization of large DNA fragments and *in vivo* to enhance plasmid stability.
4. To develop methods for selecting specific large clones containing DNA from a region of interest.
5. To incorporate in the vector design features which will assist in the mapping of restriction sites in the large pieces of cloned DNA.
6. Ultimately, to construct comprehensive libraries of large DNA fragments from mouse and human DNA.

Development of Vectors Based on the Plasmid Replicon of Bacteriophage P1 for Propagating Large Clones in *Escherichia coli*

Though large fragments of foreign DNA have been maintained in yeast as minichromosomes, only recently has progress been made toward developing such systems for *E. coli*. The main goal of this work will be to develop a system for cloning and propagating large DNA fragments in *E. coli*. Ultimately, it will be useful to have several host-vector systems that are capable of maintaining large DNAs since some DNA sequences may be clonable in one system but not in another. In addition, methods for introducing foreign DNA into yeast are at least three orders of magnitude less efficient than those available in *E. coli*, making the bacterial system the one of choice for attempting to generate comprehensive large fragment libraries.

Bacteriophage P1 is a temperate phage of *E. coli*. The P1 prophage is a plasmid, i.e., a self-replicating, circular DNA molecule. It is large, about 100 kbp, but the plasmid prophage is very stable; its rate of loss is estimated as being less than 1 in 10^5 per generation .

An important part of developing a system for cloning large DNA fragments into bacteria is finding conditions for introducing these DNA fragments into

bacteria. Several such systems are available including packaging the DNA into phage heads, for example, lambda clones or cosmids, DNA mediated transformation, or electroporation. Packaging into infectious phage heads could probably not accommodate more than 100-200kbp of DNA based on the capacity of the largest bacteriophage heads. Though traditional wisdom suggests that transformation becomes less efficient as the size of the transforming DNA increases, we have preliminary results using intact P1 prophage (about 95 kbp) which suggest that a reasonable transformation efficiency (10^5 to 10^6 transformants per μg of input DNA) can be obtained using standard transformation protocols (Dikas and Mural, unpublished). Bearing in mind that a microgram of 100kb length DNA contains about 5×10^9 molecules, this means that between .01 and .1% of the molecules are being recovered. This level may in fact be adequate for this project. What the true upper size limit, if any, may be for transformation with DNA will be determined only as we gain experience in building large DNA clones. We are currently exploring electroporation as a means of introducing large DNA's into *E. coli* and though the system is not yet optimized, we have already obtained more than 10^6 transformants per microgram of input DNA with P1 sized molecules.

Several aspects of the proposed cloning system have been tested using a simplified vector. The two components of the vector were assembled from pHSS6 and pOX38::mTn. Each component contains a loxP site, a unique NotI site, a second unique site to create an incompatible end, and an antibiotic resistance gene.

To simplify construction, the plasmid origin pMB9 was used rather than the P1 rep-par fragment, which will ultimately be included. The kan-ori fragment was prepared directly by NotI-SalI digestion, while the amp fragment was separated from its origin (not shown) after NotI-BamHI digestion. To prevent vector-vector ligation, the fragments were dephosphorylated.

To test the ligation and lox-cre mediated circularization system, an 8 kbp EagI fragment of yeast DNA was prepared as a test insert. The vector1-insert-vector2 fragments were mixed at the ratio 3:2:3 and ligated at a total DNA concentration of 100 μg/ml. Examination of the ligation products indicated complete ligation of the insert, with some residual vector fragments remaining unligated. A portion of the ligation mixture (50 ng total DNA) was treated with 1 unit of Cre recombinase which catalyzes recombination at the loxP site leading to formation of circular DNA. The untreated and Cre-treated ligations were introduced into bacteria by conventional transformation as well as by electroporation. Transformants were selected by plating on kan-amp medium. Cre-treatment resulted in 30-fold stimulation of double resistant transformants, of which 75% had the test insert in the predicted plasmid structure.

DNA REPAIR AND MUTAGENESIS

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DNA repair is a universal and vital phenomenon. It is essential for maintaining the integrity of the genome, which is subject to alterations, both spontaneous, and induced by diverse chemical and physical agents. The repair is neither perfect nor complete. The persistence of unrepaired lesions as well as error-prone repair lead to mutations, tumors and toxicity. In this program, our involvement in both DNA repair and mammalian mutagenesis is summarized as follows.

DNA Repair

Our work is exclusively focused on the repair of alkylation damage in DNA in human and mammalian cells. Simple alkylating agents induce two major types of DNA adducts, namely O^6-alkylguanine, a mutagenic and possibly carcinogenic lesion, and *N*-alkylpurines (mostly 3-alkylguanine, 3-alkyladenine and 7-alkylguanine), which are likely to be toxic if not repaired. O^6-alkylguanine in DNA is repaired by *in situ* dealkylation in a stoichiometric reaction in which the repair protein, O^6-methylguanine-DNA methyltransferase (MGMT), accepts the alkyl group on one of its cysteine residues. *N*-alkylpurines are removed catalytically by *N*-methylpurine-DNA glycosylase (MPG) leaving abasic (AP) sites in DNA which, if not processed further, are likely to be mutagenic and toxic. Both MGMT and MPG are well regulated in mammals just as in bacteria, although the phenomenology of MPG regulation has not been extensively studied. An understanding of the molecular basis of regulation of these proteins will help in identifying the factors that affect both somatic and germ cell mutations. This information has also practical implications in that MGMT expressed in certain tumor cells antagonizes alkylating drugs. Our efforts over the last few years in cloning the cDNA and the gene of human MGMT had been unsuccessful (as was also the case for a number of investigators elsewhere) until we attempted a novel approach which is based on phenotypic rescue of *E. coli*. We have now cloned the cDNA of human MGMT from a cDNA expression library and characterized it. The active human MGMT expressed in *E. coli* has a molecular weight of 22 kDa, in agreement with our earlier studies with MGMT purified from human placenta and as predicted from an open reading frame of the cDNA. The cDNA did not hybridize with DNAs from *E. coli* and yeast but has homologous

sequences in rodent DNAs. The Mex^- cell lines in which MGMT activity is absent do contain normal MGMT gene sequences. However, these cell lines do not express the 0.95 kb poly(A)$^+$ transcript of the gene.

Using an analogous approach, we have also succeeded in cloning the human MPG cDNA. Current experiments are directed toward characterization of the cDNA as well as cloning and characterization of MGMT and MPG genes of human and mouse.

Mutagenesis

Our mutagenesis studies involve ionizing radiations of both low (X ray) and high (α particle) LET types as mutagens as well as mutations induced by site-specific insertion of alkylated bases in a target DNA. In last year's report we described the shuttle plasmid system used as a surrogate for studying human mutagenesis. We have continued to use the plasmid pZ189 for determining the yield and type of such mutations induced by X rays and α particles. These studies are extremely important in view of the paucity of information on the molecular bases of mutations in general and on radiation-induced mutations in humans, in particular. The availability of a small target in the plasmid pZ189, which allows phenotypic screening for mutation, has enabled us to quantitate mutational yields following *in vitro* irradiation of plasmid DNA with X rays and α particles and to determine mutation distribution at the nucleotide level. The disadvantage of the small target is that mutations involving large-scale sequence changes cannot be identified. At the same time, such targets provide the major advantage that the data on the molecular nature of mutations, not available so far, can be readily obtained.

Analysis of more than a hundred independent mutations in the *supF* target of pZ189 shows that (1) the distribution of mutation is highly nonrandom, (2) the mutation frequency increases in a dose-dependent manner after irradiation with X rays and α particles, (3) the mutational spectra are remarkably similar in control and X-irradiated plasmids, and (4) the most common mutations are G•C→A•T transitions and in the doublet 5' TC (3' AG) sequences. The qualitative identity of mutations in control- and treated-plasmids suggests that similar mutagenic lesions are produced, possibly due to oxidative damage, during DNA transport in human cells and after X-irradiation.

The human plasmid is also being used for studying the parameters that affect mutations induced by alkylated bases. The plasmid pZ189 has been modified such that a unique *Xho*I site has been introduced and an *Ava*I site in a dispensable region has been abolished. A 145 bp *Ava*I/*Xho*I fragment of the *sup*F target can now be removed and substituted with a corresponding synthetic

oligonucleotide in which guanine in different locations will be substituted with O^6-alkylguanine in the first series of experiments. With this experimental approach we should be able to identify not only the various mutations induced by a specific mutagenic lesion but also the factors that influence such mutations.

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STRUCTURE AND ORGANIZATION OF A EUKARYOTIC GENOME WITH EMPHASIS ON SATELLITE DNAs, AND THE MOLECULAR BIOLOGY OF CRUSTACEAN MOLTING

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We are defining the organization and function of very highly repeated DNAs in the genome of a higher eukaryote, the Bermuda land crab, *Gecarcinus lateralis*. Under present study is one such satellite DNA of very complex sequence. Each 2.1 kb repeat is comprised of two distinctly different classes of domains. Six highly conserved domains that range in size from ~560 bp to 54 bp have 85-96% identity among three cloned variants that have been

completely sequenced, among those segments of nine other variants that have been sequenced, and among satellite inserts in two cDNA clones. The conserved domains comprise 70% of each repeat unit; their sequences are typical of those that harbor genetic information. The conserved domains are interspersed with four divergent domains, each composed of a family of distinctive, repeated sequences, but each unrelated to the other three divergent domains. A fifth domain between conserved domains 5 and 6 is rich in alternating purines and pyrimidines (Pu/Py). Several of the divergent domains and the Pu/Py-rich domain contain unusual primary sequences that can adopt unusual higher order structures characteristic of transcription regulatory elements and/or hotspots for mutations. In surveying the model system, we relate it to the biologically pertinent events of the model animal's changing life cycle.

Transcription of a Complex Eukaryotic Satellite

To explore possible biological functions of the satellite, we are characterizing its transcription. We use as probes specific fragments each of which contains one of the longest conserved regions of the satellite. Discrete satellite transcripts detected in total RNA from digestive gland, muscle, gill, and ovary range in size from 2.5 to 0.3 kb. Some satellite transcripts identified in poly(A)$^+$RNA are DNA strand- and tissue-specific; several tissues contain transcripts of one or the other strand. Transcription of the satellite also appears to be related to the animal's intermolt cycle; there are marked decreases in the abundance of three transcripts ranging in size from 2.5 to 1.1 kb in poly(A)$^+$RNA from claw muscle of postmolt as compared to premolt crabs. The satellite sequence is also transcribed in other crustaceans including two other species of crabs, a shrimp, and the American lobster. Transcripts of AMPL, a 142 bp segment which is single copy in most satellite variants but amplified as much as 50-fold in some, are detectable in poly(A)$^+$RNA but not total RNA of land crabs; they occur in abundance in total RNA of the lobster. Our results thus far indicate that the transcription of the AMPL segment of this complex satellite is both tissue- and DNA-strand-specific. The satellite is likely to have a biological role since its transcription occurs in a wide range of crustacean species.

Further evidence for transcription of segments of the satellite comes from clones containing satellite sequences that have been identified in a cDNA library we have constructed from poly(A)$^+$RNA from regenerating limb buds. One clone contains the AMPL segment, the other clone contains the 3' end of the satellite. We are characterizing and sequencing these clones.

Unusual Higher Order Structures Assumed by Satellite Sequences

Plasmids containing pyrimidine • purine tracts can form both inter- and intramolecular triplexes. Addition of poly(dTC) to plasmid pTC45, which contains a (TC)45•(GA)45 insert, results in intermolecular triplex formation. Agarose-gel electrophoresis gives rise to many well-resolved bands, which correspond to 1, 2, 3, 4... plasmid molecules attached to the added pyrimidine strand. In the electron microscope these complexes appear as a rosette of petals. The mobility of these triplex-containing complexes can be retarded by the addition of a triplex-specific monoclonal antibody, Jel318. Intramolecular triplex formation can be demonstrated at pH 5 in pTC45 and also in pT463-I, a plasmid containing a segment of the crab satellite with both (G)n•(C)n and (TCC)n•(GGA)n inserts. Although the intermolecular triplex remains stable at pH 8, intramolecular triplex formation only occurs at low pH. Triplexes can also be detected by an immunoblotting procedure with Jel318. This unfamiliar triplex structure is readily demonstrated in eukaryotic extracts, but not in cells from *E. coli*. Triplexes may thus be an inherent feature of eukaryotic chromosome structure.

The Molecular Biology of Crustacean Molting

Proteins and proteinases of the integumentary tissues and exoskeleton of the land crab are also under investigation. Some proteins are specific to one of the four exoskeletal layers while others are common to several layers. All layers of the intermolt exoskeleton, except the outermost epicuticle, are rich in small M_r proteins (<28 kDa), as are insect cuticles. The innermost membranous layer, which is the site of separation of epidermis from the old exoskeleton at the onset of premolt degradation, is particularly rich in small proteins. Proteins ranging in size from ~200-300 kDa are also seen. Of 59 proteins extracted from epicuticle, exocuticle, and endocuticle of newly synthesized exoskeleton of the blue crab, *Callinectes sapidus*, 88% match proteins from similar layers of *G. lateralis* in both size and IEF point. In addition to *in vivo* and *in vitro* studies on the synthesis and degradation of these structural proteins that account for >30% of the exoskeleton, we have identified several enzymes active in degrading the exoskeleton during premolt. These are either alkaline or acid proteinases. Confirming their biological importance is the absence from the exuviae of newly molted crabs of a series of proteins of the same size as those that are degraded *in vitro* by the purified integumentary proteinases.

Proteinase activity extracted from intermolt integumentary tissues and from exuvia has been demonstrated directly in gels permeated with gelatin. Enzyme activity in such gels is identified as clear bands at the positions of specific proteinases that degrade the gelatin substrate. Bands of similar sizes are seen in exuvia and in partially purified enzyme extracts, verifying the biological role of

the enzyme extracts. The ability of Ca^{++} to bind to exoskeleton proteins and its role in proteinase activation are under investigation.

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STRUCTURAL BIOLOGY OF NUCLEOSOMES AND GENES

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This laboratory is investigating the structure of macromolecular components in genetic material. The goal of this research program is to understand the structural organization, function, and dynamic behavior of the nucleosome, DNA, histones, chromatin, and other macromolecules in relation to control of gene expression.

Nucleosome Core Particle Crystal Structure

The nucleosome core particle structure has been determined to a resolution of 8 Å by X-ray crystallographic methods. Features present in this structure include the histone organization, the double helical DNA structure, numerous protein-DNA interactions, and distortions in the path of the DNA around the protein core.

The DNA in the nucleosome core particle bends much more substantially than any other DNA structure solved by crystallographic methods. The size and shape of the DNA grooves provide evidence of the distortions in the B-DNA structure which facilitate DNA bending. These measurements provide insight into the mechanisms of histone-induced DNA bending, the sequence dependence of histone binding, and the helical parameters of nucleosomal DNA. Our research indicates that the bending of DNA is accomplished by considerable compression of the major grooves on the inner surface next to the protein core and expansion on the outside of the superhelix. We have also detected a structural motif in the DNA with a period of two B-DNA helical repeats, or about 20 base pairs. This feature results from the placement of AA and TT dinucleotides at specific positions within the 20 base pair repeat, and these dinucleotides modulate the DNA structure for optimum major and minor groove bending around the histone core. The level of detail in the histone portion of the structure at 8 Å resolution permits a more accurate assignment of histone domains and clarification of the overall organization of the histone core. This information enables a detailed comparison to other related structures and an evaluation of flexible regions of the histones. The histone structure suggests possible mechanisms of unfolding the nucleosome particle which could be related to control of gene expression. Analysis of the histone areas associated with contacts between nucleosomes in the crystal structure suggests possible modes of interaction between nucleosomes in chromatin higher order structure.

Nucleosomes reconstituted from homogeneous histone octamers and cloned specific-sequence DNA are necessary in order to determine a high resolution (~ 3 Å) crystal structure. Several DNAs that form precisely positioned nucleosomes are being developed. These sequences are a repeat unit subcloned from human α-Satellite, a sequence from SV40 flanking the nucleosome free region, and a chicken t-RNA lysine gene complete with its polymerase promotor region. The sequences chosen for the high resolution crystallographic studies exhibit strong positional preference on nucleosomes *in vivo* as well as by predictive algorithms. Fourier analysis of DNase I protection patterns demonstrate that precise reconstitutions have been achieved for all three sequences in our laboratory. A 163 base pair SV40 DNA subclone is centered on the nucleosome at base pair 343, in good agreement with the predicted

center at base pair 341.5. The predicted center for the α-Satellite DNA on the nucleosome is at base pair 1310, and the experimentally determined center is at base pair 1309.5. The *in vivo* t-RNA^{lys} gene is centered at base pair 263. Our theoretical prediction is in agreement with the experimental determination.

The high resolution structure will be phased using multiple energy anomalous dispersion data collected at the Brookhaven National Synchrotron Light Source. It will be possible to trace the peptide chains of the histone molecules and the phosphodiester backbone of the DNA, and locate the DNA bases in the resulting electron density map. This long term research is providing information to understand control of gene expression at the nucleosomal level.

Properties of Precisely Positioned Nucleosomes

The positional preferences of dinucleotide types in precisely positioned nucleosomes have been analyzed by statistical methods and algorithms developed for the prediction of nucleosome positions along DNA. Analyses of the DNA sequence patterns in precisely positioned nucleosomes revealed the existence of several DNA sequence periodicities including 6-7, 10, and 21 base pairs. The results demonstrate that each dinucleotide type is unique in terms of its positional preference in precisely positioned nucleosomes, and that the sequences adjacent to major groove-in sites are of paramount importance for bending into the major groove.

The significance of AA/TT pairs in nucleosomes compared to solution curvature of DNA has been investigated by analyzing the patterns of dinucleotide occurrences within the average helical repeat of the DNA sequences. Unlike DNA curvature in solution, which occurs by compression of AA/TT into the minor groove, nucleosomal DNA is bent primarily by compression of the major groove. The DNA sequences that form precisely positioned nucleosomes exclude AA/TT pairs from occupying major groove-in locations with one specific exception located at position $\pm$ 30.

Since AA/TT pairs perhaps stiffen the DNA or promote curvature toward the minor groove, the primary constraint for nucleosome formation is to avoid AA/TT placement at major groove-in sites where they would prevent the histone core from bending the DNA. Therefore, AA/TT pairs are not the primary factor for bending nucleosomal DNA, but by their absence at major groove-in locations the DNA can be bent more easily by the histone core, thereby affecting the overall positioning of the DNA on the nucleosome. The positional preferences of AA and TT dinucleotides used with the roll/tilt wedge model of DNA bending predict the 20 base pair structural repeat observed in our nucleosome core particle crystal structure.

Protein Structure Determination

O^6-Alkylguanine lesions in DNA are repaired by *in situ* dealkylation in a stoichiometric suicide reaction by O^6-Methylguanine-DNA methyltransferase which accepts the alkyl group in one of its cysteine residues. Studies are in progress to obtain crystals of this repair enzyme suitable for X-ray crystal structure determination.

Yeast inorganic pyrophosphatase is a dimeric 64 kDa enzyme that catalyzes the hydrolysis of pyrophosphate. This highly exergonic reaction drives to completion numerous otherwise reversible inorganic pyrophosphate-yielding reactions including nucleic acid biosynthesis and fatty acid biosynthesis. Pyrophosphatase is therefore an essential enzyme in all life forms. The high resolution, low temperature structure of yeast inorganic pyrophosphatase is being refined in collaboration with D. Voet at the University of Pennsylvania.

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ENZYMES INVOLVED IN RNA TURNOVER AND SYNTHESIS

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Cloning and Characterization of a 5'→3' Exoribonuclease Gene of *Saccharomyces cerevisiae*

Regulation of the cytoplasmic stability of mRNAs has been found to be a major control mechanism which regulates mRNA levels in a variety of eukaryotic and prokaryotic systems. An important part of unravelling the mystery of mRNA turnover is to analyze in detail the enzymes that may be involved. RNA processing reactions which convert precursor RNA species to mature RNA molecules also involve unique ribonucleases. Studies of ribonucleases and their possible role in RNA turnover and processing have been continued in this laboratory.

A gt11 yeast genomic library was screened for specific antigen-producing clones using rabbit polyclonal antiserum for a 5'→3' exoribonuclease of *Saccharomyces cerevisiae*. Of the positive clones identified, four were examined in detail by restriction enzyme mapping and analyses of the cloned DNA fragments by Southern and Northern analyses. Cross-hybridization studies showed that two of the clones contained overlapping DNA sequences. An EcoR1 restriction fragment common to the two clones hybridized by Northern analyses with a 5 kb mRNA, which agrees with the size calculated on the basis of the size (160 kDa) of the exoribonuclease. Southern hybridizations were carried out using the cloned restriction fragment as a probe for yeast genomic DNA separately digested with several restriction endonucleases. The appearance of a single band per digest indicated that the probe hybridizes to a unique sequence. The fragment was also used as a probe to screen the Carlson/Botstein YEp24 yeast genomic library by colony hybridization in order to obtain a full-length genomic clone of the exoribonuclease gene. Two positive YEp24 clones were obtained which contained inserts of about 12 kb in length. Overexpression of the exoribonuclease in yeast was found with both the YEp24 clones. The overexpression was determined by both Western antibody analyses and by direct assays of enzyme activity. A four- to six-fold higher level of enzyme was found. Restriction digests and deletion mapping of the two YEp24 clones allowed a fragment of about 7 kb to be identified as containing the exoribonuclease gene. Experiments are now in progress to disrupt the gene and analyze the results of the disruption.

Purification of Poly(A) Polymerase I of *Saccharomyces cerevisiae*

Poly(A) polymerase is an enzyme involved in polyadenylation of mRNA, and its role may be very important both by its playing an integral role in the synthesis of mRNA and by its causing increased stability of mRNA. More than one poly(A) polymerase is found in eukaryotic cells and the precise roles of each of these enzymes is not known. Toward the end of cloning the gene for the enzyme and testing its essentiality, poly(A) polymerase I of *S. cerevisiae* has been purified 1000-fold by column chromatography on DEAE, phosphocellulose, heparin-agarose, and DNA cellulose. By gel analysis, the enzyme is about 10% pure and is a 66 kDa protein. Antiserum production and amino acid sequence analysis using gel bands are in progress so that cloning of the gene can be initiated.

Phosphorylation of RNA Polymerase II

The C-terminal domain of the large subunit of eukaryotic RNA polymerase II consists of multiple repeats (52 in mammals) of a heptapeptide with the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. Studies of others show that

the sequence is essential *in vivo* and that a form of the enzyme in which the C-terminal sequence of the large subunit is highly phosphorylated is the transcriptionally active form in HeLa nuclei.

In a search for protein kinase activity that could play an important regulatory role involving RNA polymerase II phosphorylation, we have used as a substrate a synthetic peptide, Lys(Tyr-Ser-Pro-Thr-Ser-Pro-Ser)$_4$, homologous to the C-terminal repeated sequence of the large subunit of RNA polymerase II. We find that HeLa cell extracts and fractions derived therefrom contain two peptide-phosphorylating activities which can be distinguished from casein kinases I and II, protein kinase C, and cAMP-dependent protein kinase (the latter enzymes do not phosphorylate the peptide). One of the enzymes, which has been purified about 200- to 300-fold, is inhibited by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), an inhibitor of mRNA synthesis in mammalian cells, and is immunoprecipitated by an antibody against the cell-cycle control protein cdc2. (The latter finding is analogous to a report by others on the phosphorylation of RNA polymerase by the murine homologue of the cdc2 protein.) Characterization of the complete subunit composition, substrate specificity, and the DRB inhibition kinetics of the enzyme is in progress. The second enzyme accounts for more than 50% of the peptide-phosphorylating activity, but is not inhibited by DRB and not immunoprecipitated by the cdc2 antibody. Further purification of the enzyme is in progress.

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1. Stevens, A., and M. K. Maupin. 5,6-Dichloro-1-β-D-ribofuranosyl-benzimidazole inhibits a HeLa protein kinase that phosphorylates an RNA polymerase II-derived peptide. *Biochem. Biophys. Res. Commun.* **159**: 508-515, 1989.

REGULATION OF MAMMALIAN-CELL TRANSPORT SYSTEMS

J. S. Cook
M. H. Bast[5]
P. J. Galloway[5]
M. R. Hauser[1]
B. G. Stanford

The transport of metabolites across cell surfaces or across epithelial sheets of cells is studied by this group in mammalian cell systems. Cloned cells in culture are used because they insure physiological and genetic homogeneity as well as experimental control over the chemical environment (hormones, growth factors), thus permitting the unambiguous identification of cell types and the environmental factors to which they respond. Recent work has focused on a cell

line derived from pig kidney, LLC-PK_1, that differentiates *in vitro* into a population with the properties of proximal tubule cells, and on fibroblast lines responsive to epidermal growth factor (EGF). The goal of this group is to develop an understanding of the regulation of specific transport systems in relation to cell growth and differentiation. Four projects are outlined for this report period:

Protein Kinase C and the Regulation of Amino Acid Transport

Confluent LLC-PK_1 cells depleted of endogenous amino acids display a biphasic response of Na^+-dependent amino acid transport (A-system) to the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA); the early response corresponds to a TPA-induced translocation of protein kinase C from cytosol to the membrane and an increase in membrane protein phosphorylation. Both transport and phosphorylation peak within 5-10 min after TPA exposure and return to near control levels for the next 20-30 min. The increases in transport and phosphorylation can both be inhibited by sphingosine, an inhibitor of protein kinase C, and are prolonged in the presence of NaN_3, a phosphatase inhibitor that has no direct effect on the transporter. Several membrane-associated proteins show a rise and fall of phosphorylation in response to TPA and sensitivities to sphingosine and NaN_3 corresponding to the observed transport changes. Similar rapid responses are elicited by diglycerides that also activate protein kinase C and stimulate the A-system. In confluent cultures sphingosine inhibits only the TPA-sensitive portion of A-system transport but not the basal rate. Conversely, in rapidly growing cultures in which the kinase is constitutively membrane-associated and which have a >15-fold higher rate of transport, TPA is without effect and sphingosine inhibits the A-system by 50%. The results suggest that membrane-associated protein kinase C maintains the transporter or a transport regulator in a phosphorylated, active state in growing cells, but dissociates from the membrane at confluence, after which time the regulators are dephosphorylated; TPA can reverse this differentiation-related distribution.

Firm conclusions from these results may be compromised by the long-term toxicity of sphingosine. We have therefore looked for other protein kinase C inhibitors that can permeate cells without toxic effects. Three diphenolic compounds, quercetin, phloretin, and diethylstilbestrol, have been found to meet these criteria. Their effects on TPA-stimulated upregulation of the A-system are the same as those of sphingosine and confirm our original interpretation.

Differential Expression of Genes for Hexose Transport in Differentiating Kidney Cells

At the apical (luminal) surface of differentiated LLC-PK_1 cells is a Na^+-coupled glucose transporter (NaGT) which co-transports Na^+ and hexose. The

electrochemical driving force on Na^+ from lumen to cell interior can energize the NaGT to drive glucose against its chemical gradient to high levels. Once in the cell, glucose exits over the basolateral Na^+-independent glucose transporter (GT). In parallel, the Na^+/K^+-ATPAse at the basolateral surface extrudes the co-transported Na^+, maintaining the Na^+ gradient.

Several literature reports have led us to the following working hypothesis: in proliferating LLC-PK_1 cells a low K_m, high affinity, GT is expressed that is functionally adapted to the cells' need for glucose as a metabolite; for these cells glucose is needed to support growth, and the hexose that is taken up is immediately phosphorylated and enters the metabolic pathways and is not again released as native glucose. In differentiated cells expressing the apical NaGT, on the other hand, the basolateral GT is a high K_m, low affinity transporter functionally adapted to allowing free glucose to exit from the basolateral surface, thereby accomplishing transepithelial transport. We have obtained kinetic transport evidence that supports the low K_m-to-high K_m shift on differentiation. We also have obtained from Harvey Lodish (MIT) suitable cDNA probes with which we are exploring this shift in gene expression at the mRNA level. Both GT genes are present in the genomic DNA of LLC-PK_1 cells (Southern blot analysis) and the patterns of mRNA expression are compatible with the hypothesized shift (Northern blot analysis). The detailed temporal relationship in comparison with the (co-)expression of the NaGT, and the sensitivity of mRNA expression to transport modifiers like tumor promoters and epidermal growth factor, are under investigation.

Transport of Polyamines

Polyamines are a group of compounds including putrescine, spermidine, and spermine, containing two to four amino groups. They are required for cell growth and differentiation, possibly through an interaction with exposed phosphate groups in DNA. In LLC-PK_1, polyamines are taken up by transporters that are separate from the well-known renal transporter for organic bases. The two polyamine transporters are distinguished in that one is Na^+-dependent and one Na^+-independent, the former with a K_m of ~10 μM, which is in the physiological range of the blood level. In differentiated renal cells, putrescine is either transported across the sheet of cells in a direction suggesting that *in vivo* it is recovered from the glomerular filtrate and restored to circulating blood plasma, or it is oxidatively deaminated to form γ-aminobutyraldehyde; very little is metabolized to the higher polyamines. A provocative finding is that the aminoglycoside antibiotics gentamicin, neomycin, and kanamycin, clinically used antibiotics that are nevertheless toxic to the kidney *in vivo*, compete with and can block polyamine uptake by kidney cells.

Cellular Responses to EGF Mutants

We have most recently been exploring the responses of Balb c/3T3, strain A31, cells to mutants of human epidermal growth factor (hEGF), engineered by site-directed mutagenesis in the laboratory of S. K. Niyogi. These mutants have been characterized by Niyogi's group with respect to their binding to EGF receptors *in vitro* and their effects on the V_{max} of the receptors' tyrosine kinase activity. We have been extending these studies to the cell biological responses, focusing initially on stimulation of [^{3}H]thymidine incorporation into DNA and the phosphorylation of cellular proteins *in vivo*. In general, we find that the cells respond in the direction expected from the biochemical studies, i.e., poorly binding mutants are less effective and tightly binding mutants more effective than native hEGF when assayed on whole cells, but the quantitative differences in effectiveness are much less than those observed *in vitro*, more than two orders of magnitude in some cases. At sufficient concentrations, mutants that stimulate tyrosine kinase activity to a lower V_{max} *in vitro* can nevertheless elicit the maximum degree of [^{3}H]thymidine incorporation in intact cells. The quantitative differences between the *in vivo* and *in vitro* studies appear to be rate effects related to differences in kinetics of binding to the receptor or to the rate of signal transduction, hypotheses that are still being explored. We plan to extend these studies to other responses of cells to hEGF, including phospholipase C activation (seconds), Na^+/H^+ antiport stimulation (minutes), and induction of new mRNA for hexose transporters (hours).

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1. Hauser, M.R. and J. S. Cook Uptake and metabolism of putrescine in confluent LLC-PK$_1$ cells. *Am. J. Physiol.:Cell Physiol.*, in press.

THEORETICAL AND APPLIED CRYOBIOLOGY

P. Mazur

K. W. Cole
J. W. Hall

Basic Mechanisms of Slow Freezing Injury

The causes of slow freezing injury are the subject of debate. During slow freezing, cells are sequestered in unfrozen channels between ice crystals that grow by pulling pure water out of the channels. As a consequence, the solute concentration in the channels rises and the channels progressively shrink in size. The rise in solute concentration, in turn, causes the cells to progressively shrink osmotically. Until recently cryobiologists have ascribed slow freezing injury either

to the rise in solute (electrolyte) concentrations or to the consequent cell shrinkage, rather than to the size of the channels. But we have evidence in human red blood cells that the latter is an equally important cause of injury. One line of evidence is the following: Although ordinarily reciprocally coupled, it is possible to separate the composition of the unfrozen channels from their size by suspending cells in NaCl/cryoprotectant solutions in which the mole ratio of the two is held constant, but the molality of the NaCl is allowed to vary below and above isotonic. When human red cells are frozen in such solutions to temperatures that produce given NaCl concentrations (m_s), but varying unfrozen fractions (U), survival at low U is found to be strongly dependent on U but independent of m_s. Only at higher values of U, does survival become inversely dependent on both m_s and U.

Although this approach separates m_s and U, it of necessity introduces confounding from another source; namely, when the initial molality of NaCl is varied, the cells have different volumes at the onset of freezing. From room-temperature dialysis experiments mimicking the concentrations occurring during freezing, D. Pegg argues that it is these differences in initial cell volume, and not differences in the unfrozen fraction attained, that cause injury. But we have counter-evidence from experiments involving the subjecting of red cells to similar solute concentratoins and therefore similar volume changes during cooling to -16°C, in one case by normal freezing and in the other by avoiding any ice, that the presence or absence of ice is the main determinant of injury.

One possible resolution to the apparent paradox is that in both the dialysis experiments and freezing experiments, shrunken cells are crowded together. These are conditions that can produce membrane-membrane fusion; and indeed there is direct evidence in liposomes and indirect evidence in some cells that freezing does induce membrane fusion. We plan to look into this matter both in human red cells and bovine and human sperm. An attractive reason for using sperm is that this cell type normally becomes primed to undergo membrane fusion reactions; namely, the acrosome reaction.

Permeabilization and Cryobiology of Drosophila Embryos

Paradoxically, although mammalian embryos normally exist in closely controlled environments and insect eggs are subject to the vagueries of the environment, the former can now be frozen with high survival, whereas the latter in general cannot. With support from a grant from the National Science Foundation and in collaboration with A. P. Mahowald of Case Western University, we are investigating this paradox with *Drosophila*, one reason being the importance to *Drosophila* geneticists of developing a method for the cryopreservation of some of the ~10,000 mutant lines of this insect.

One fundamental way in which the *Drosophila* egg differs from the mouse egg is in possessing a waxy layer in the vitelline membrane that allows vapor exchange but not liquid water or solutes. Since successful freezing requires protective solutes like glycerol inside the cell and the osmotic efflux of water during freezing, that waxy layer must be removed. Sporadic reports indicate that that can be done by a brief exposure to alkanes like octane. But in our hands the problem has been exceedingly vexing. Until recently, conditions that effectively permeabilize the eggs kill them, and supposedly replicate runs yielded highly variable results. We have just found that the source of the problem is that minute amounts (>0.2%) of alcohol must be in the alkane to achieve effective permeabilization but if more than minute amounts are present (>0.4%), the alkane-alcohol mixture becomes rapidly lethal.

As mentioned in last year's report, even in the absence of freezing, the eggs become increasingly sensitive to progressively lower subzero temperatures. Thus, at 0°, -9°, -15°, -20°, and -25°C, half of 15 h eggs are killed in 37 h, 4 h, 40 min, 15', and 6', respectively. Younger eggs are still more chill sensitive. This high chill sensitivity means that orthodox slow freezing procedures to remove intracellular freezable water before cooling to the egg ice nucleation temperature of -28° to -31°C will not succeed. Success requires preventing both the low temperature injury and intracellular freezing by cooling the eggs extremely rapidly to temperatures below which any adverse reactions can occur. The cooling rate required to outrun the chilling injury depends critically on the subzero temperature at which the rate of chilling injury decreases towards 0. Thus, computations indicate that if chilling injury abruptly ceases at -70°C, attaining 50% survival will require cooling at 10,000°C/min to that temperature. There are indications from meeting reports both that a very rapid cooling technique will succeed and that the required cooling rates are on the order of 10,000°C/min.

Fundamental Cryobiology of Human and Bovine Spermatozoa

Although sperm were among the first mammalian cells to be successfully frozen, and frozen bull sperm is a major component of the large artificial insemination (AI) industry, surprisingly little is known about the fundamental cryobiology of these important cells. In this reporting period, Dr. John Critser of Methodist Hospital of Indiana and I have been awarded two grants from the NIH and the USDA to study this matter in human and bovine spermatozoa, respectively. Interest in the freezing of human sperm has been recently reinforced by the nearly mandatory requirement that human donor sperm be held > 6 months prior to AI to ensure that the donor sperm does not contain HIV. Present empirical methods of freezing bull and human sperm result in only some 25-50% functional survival.

Knowledge of the permeability of a cell to water and to cryoprotectants can be powerful tools in predicting the likely optimum values for the major steps involved in freezing. From the value of the permeability coefficient for the cryoprotectant, one can compute the optimum procedure for adding and removing the cryoprotectant without osmotic shock. From knowledge of the permeability of the cell to water and its temperature coefficient, one can predict the cooling rate likely to be low enough to preclude lethal intracellular freezing. Accordingly, the first step of our proposed research is to determine the osmotic and permeability characteristics of bovine and human sperm to water and glycerol at temperatures between 0° and 20°C. To determine permeabilities, use will be made of the fact that sperm in solutions of permeating cryoprotectant in hypotonic NaCl, like erythrocytes, swell without change in surface area to a critical volume, at which point they lyse. As with erythrocytes, the time taken to produce spermolysis is a measure of the permeability of the cell to water and the solutes in question.

Mouse Embryo Banking

There are several reasons to preserve mouse genetic stocks in the form of frozen embryos: (1) as insurance in case the breeding stock is lost by reproductive failure, disease, or fire (which occurred at Jackson Labs in 1947 and again this past year); (2) to study, control, or reduce genetic drift in inbred lines; (3) to permit experiments that otherwise could not be undertaken, such as a comparison on the same day between a pair of monozygotic twins that differ in age; and (4) to provide a method of maintaining genetic stocks that is cheaper than conventional breeding in terms of manpower, space, and facilities. In this reporting period we have been engaged in four mouse embryo banking projects funded by other agencies or institutions. One, funded by the National Institutes of Child Health and Human Development, involved the freezing of some 2000 mouse embryos in the 8-cell precompaction stage. It is completed. The second, funded by the National Institute on Aging, involves the banking of embryos from special inbred lines maintained by NIA. The remaining two, funded by two grants from the National Institute on Alcohol and Alcohol Abuse to Dr. John Crabbe at Oregon Health Sciences University, involve the banking of mouse embryos at various stages in the genetic selection of lines exhibiting various sorts of behavioral abnormalities in response to alcohol. These projects constitute an example of the transfer of a technology (i.e., mouse embryo freezing) first developed in this laboratory in 1972.

The Mammalian Genetics and Development Section also has an ongoing program of the banking of embryos of many of the genetic lines maintained by L. B. Russell. We consult with and advise F. Stenglein, who is in charge of that freezing program.

1. Mazur, P. Stopping biological time - the freezing of living cells. *Ann. N. Y. Acad. Sci.* **541**: 514-131, 1988.
2. Mazur, P. Frozen neanderthals. *Nature* **342**: 23, 1989.
3. Mazur, P., and K. W. Cole. Roles of unfrozen fraction, salt concentration, and changes in cell volume in the survival of frozen human erythrocytes. *Cryobiology* **26**: 1-29, 1989.

EDUCATIONAL ACTIVITIES

Doctoral and Postdoctoral Training Programs

The University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences was established in 1965 through a joint effort of the Biology Division of the Oak Ridge National Laboratory and The University of Tennessee, so that the scientific talents and research facilities of the Laboratory could be utilized more fully in graduate education. The School accepted its first class of seven graduate students in the fall of 1967. Since then it has grown and developed steadily. It now has a graduate enrollment of 28 students and is a recognized center of quality education in the southeast. The University of Tennessee supports 16 students with research assistanships, 8 students are supported by a National Cancer Institute Predoctoral Training Grant in Carcinogenesis Research (F. T. Kenney, PI), and 1 student is supported by a National Institute of Health predoctoral appointment. By December of 1989, a total of 125 students have been awarded Ph.D. degrees through the Graduate School of Biomedical Sciences. Nearly all continue today in careers in research or research/teaching, and some have won distinction for their research contributions. Although intended primarily for Ph.D. training, the School has awarded a total of 20 M.S. degrees. This component of the program was added to meet the needs of UT and ORNL employees.

The Graduate School offers courses and laboratory experience in many aspects of biomedical science, emphasizing biochemistry and molecular biology, carcinogenesis and radiation biology, mammalian genetics and development, and structural and cellular biology. It has one full-time faculty member from The University of Tennessee (D. E. Olins) and four research professors (A. L. Olins, R. S. Foote, E. C. Uberbacher, and L.-Y. Ch'ang) supported through extramural research funds. An adjunct faculty of 24 members from the Biology Division and other divisions of the Oak Ridge National Laboratory and 1 member from the Oak Ridge Associated Universities fulfill most of the obligations for teaching and directing thesis research. The Graduate School of Biomedical Sciences is a component of the Graduate School of The University of Tennessee but is housed within the Biology Division of the Oak Ridge National Laboratory. Both classroom teaching and laboratory research training utilize Biology Division facilities. The School's Director is employed by both institutions and reports to the Dean of the Graduate School at the University, the Biology Division Director, and the Associate Director for Biomedical and Environmental Sciences at the Laboratory.

The overall objective of the Graduate School of Biomedical Sciences is to develop a high quality, multidisciplinary graduate training program in the

Biomedical Sciences. The program begins with a core curriculum that emphasizes the multidisciplinary and quantitative aspects of modern biology while providing a diversity of laboratory research experiences. Advanced students take courses and tutorials in specialized areas, participate in research seminars, and pursue dissertation research under the direction of a faculty preceptor.

The students form a very active group of investigators as reflected by 19 coauthorships, 8 as first authors, during the last 15 months. This represents a significant contribution to the productivity and excellence of ORNL's Biology Division. In return, the students receive superb guidance and training by staff members of the Biology Division.

Postdoctoral training is another important activity of the Biology Division. The program is administered by The University of Tennessee and by Oak Ridge Associated Universities. Support for 5 postdoctoral fellows was provided by a Postdoctoral Training Grant in Carcinogenesis Research from the National Cancer Institute, and 11 postdoctoral fellows were supported by a subcontract from Martin Maretta Energy Systems, Inc. After a two- or three-year period of research in the Biology Division, trainees obtained positions in universities, industries or other government laboratories.

Undergraduate Training Programs

The Biology Division participates in three undergraduate training programs: (i) Great Lakes Colleges Association/Associated Colleges of the Midwest (GLCA/ACM Science Semester), (ii) Southern Colleges University Union Science Semester (SCUU), and (iii) Oak Ridge Associated Universities Summer Student Trainee (ORAU). Under the auspices of these organizations and in cooperation with Oak Ridge National Laboratory, outstanding college juniors are offered opportunities for independent research in the life sciences. In the past 12 months, there were 11 students, possessing the educational qualifications and the potential for a successful scientific career, who spent 16 weeks (GLCA/ACM and SCUU) or 10 weeks (ORAU) performing research under the guidance of Biology Division staff members.

Although the principal purpose of the programs is to provide a training experience for the students, it often allows Division staff members an opportunity to broaden their areas of research. Upon completion of their research activities in the laboratory, students prepare a formal scientific paper and present a talk on their work. The programs, in which over 536 students have participated during the past 23 years, have received the enthusiastic endorsement of the students, their colleges, and the members of the Biology Division.

Appendices

Advisory Committee - FY 1989

Dr. Irwin Fridovich
Department of Biochemistry
Duke University Medical Center
Durham, North Carolina 27710

Dr. William J. Schull, Director
Center for Demographic & Population Genetics
The University of Texas Health Science Center
Post Office Box 20334
Houston, Texas 77225

Dr. Shirley M. Tilghman
Howard A. Prior Professor of the Life Sciences
Department of Molecular Genetics
Princeton University
Princeton, New Jersey 08544

Dr. Joseph J. Villafranca
Professor, Department of Chemistry
152 Davey Laboratory
Pennsylvania State University
University Park, Pennsylvania 16802

Dr. Arthur Weissbach
Post Office Box 168
Sanibel, Florida 33957

Extramural Activities

Society Committees

J. S. Cook - Publications Committee, American Physiological Society, 1986-1989; Chairperson, 1989-1992

R. J. M. Fry - History Committee, Radiation Research Society, 1982-

F. C. Hartman - Symposium Director, The Protein Society, 1990

P. Mazur - Publications Committee (Chairman), Society for Cryobiology, 1974-
Board of Governors, Society for Cryobiology, 1981-

A. L. Olins - Minorities Committee, American Society for Cell Biology, 1985-
Organizer of workshop, "Careers in Biological Sciences: Advancement for Women and Minorities," April, 1990 (supported by National Science Foundation)
University of Tennessee, Faculty Senate, 1989-1992

R. J. Preston - President, Environmental Mutagen Society, 1989-1990
John Sealy Memorial Endowment Fund Grants Committee, University of Texas Medical Branch, 1987-

D. M. Skinner - Board of Governors, The Crustacean Society, 1987-
Selection Committee, Miller Research Fellows, Miller Institute for Basic Research in Science, University of California, Berkeley, 1987-

Advisory Committees

J. S. Cook -
- Member of the Corporation, Mount Desert Island Biological Laboratory, 1962-
- Special Study Sections, National Institutes of Health, 1987-1989
- External Review Committee, Department of Biology, Temple University, 1988

R. J. M. Fry -
- Scientific Committee 40, National Council on Radiation Protection and Measurements, 1977-
- Council Member, National Council on Radiation Protection and Measurements, 1980-
- Scientific Committee 75 (Chairman), National Council on Radiation Protection and Measurements, 1983-
- Committee 1, International Commission on Radiological Protection, 1985-
- Advisory Committee, Institute of Environmental Medicine, New York University Medical Center, 1985-
- Committee on Space Biology and Medicine, Space Science Board, National Research Council, National Academy of Sciences, 1986-
- Member, Aerospace Medicine Advisory Committee, National Aeronautical Space Administration, 1988-
- Chairman, Environmental Sciences Working Group, National Aeronautical Space Administration, 1988-
- Member, Bevalac Biomedical Program Advisory Committee, Lawrence Berkeley Laboratory, 1988-1990
- Member, Committee on an Assessment of a Study of Possible Occupational Health Effects on Ionizing Radiation Among Nuclear Utility Workers, National Research Council, Commission on Life Sciences, 1988-

W. M. Generoso -
- Committee on Toxicology, National Research Council (National Academy of Sciences), 1989-1991

F. C. Hartman - Executive Committee, Division of Biological Chemistry, American Chemical Society, 1989-1991

S. J. Kennel - Study Section on Health Effects Research, U.S. Environmental Protection Agency, 1982-
Ad hoc member, National Cancer Institute Site Visit Committees, 1989

F. T. Kenney - Adviser, National Cancer Institute Outstanding Investigator Grants Program, 1984-

A. C. Marchok - Member, Pathology B, Study Section, National Institutes of Health, 1988-1992

S. Mitra - Consultant, National Cancer Institute, 1987-

A. L. Olins - Member of the Corporation, Woods Hole Marine Biological Laboratory, 1983-
Special Study Section, National Institutes of Health,1988, 1989

D. E. Olins - Member of the Corporation, Woods Hole Marine Biological Laboratory, 1983-
Advisory Committee, Helicon Foundation, La Jolla, California, 1983-
Ad hoc Reviewer, American Cancer Society, 1989

R. A. Popp - Mouse Hemoglobin Nomenclature, 1984-

R. J. Preston - Genetics Working Group, American National Standards Institute, 1983-
Talent Pool for Committee on Radiation Research and Policy Coordination, 1985-
Gene-Tox Panel on In-Vivo/In Vitro Cytogenetics (Chairman), Environmental Protection Agency, 1986-
Toxicology Study Section, National Institutes of Health, 1988-1992
Genetics/Molecular Biology consultant, Radiation Effects Research Foundation, Hiroshima, Japan, 1989-1991
Genetics Effects Expert, Metropolitan Edison, 1989-

L. B. Russell - International Committee on Standardized Genetic Nomenclature for Mice, 1977-
Coordinating Committee of Gene-Tox, U.S. Environmental Protection Agency, 1980-
Board on Environmental Science and Toxicology, National Academy of Sciences, 1986-1990
Environmental Health Institute, Fellow, 1987-

D. M. Skinner - Member of the Corporation, Marine Biological Laboratory, Woods Hole, 1971-
Member, Advisory Committee, Department of Biology, Georgetown University, 1983-
Panel Member, Physiological Processes Program, National Science Foundation, 1986-1989
Panel Member, Presidential Young Investigators, National Science Foundation, 1989

A. L. Stevens - Biomedical Sciences Study Section, National Institutes of Health, 1985-

W. K. Yang - Cancer Biology and Immunology Contract Review Committee, National Cancer Institute, 1987-1991
Outstanding Investigator Award Review Committee, National Cancer Institute, 1989

Editorial Boards

J. S. Cook - *American Journal of Physiology*,
Associate Editor, 1981-1987;
Acting Editor, 1984-1985;
Editorial Board 1987-1990
Current Topics in Membranes and Transport,
Advisory Board, 1983-
News in Physiological Sciences,
Editorial Board, 1985-1986
Associate Editor, 1986-
Chair, Managing Board, 1989-1992

R. J. M. Fry - *Radiation Research*, Editor-in-Chief, 1988-

W. M. Generoso - *Teratogenesis, Carcinogenesis, and Mutagenesis*, 1979-
Mutation Research, 1985-1989
Molecular Toxicology, 1986-

F. C. Hartman - *Journal of Protein Chemistry*, 1982-1992
Journal of Biological Chemistry, 1983-1988

P. Mazur - *Cryobiology*, 1967-

R. J. Preston - *Mutation Research*, 1977-
Environmental and Experimental Botany, 1979-
Cell Biology and Toxicology, 1989-
Environmental and Molecular Mutagenesis, 1989-
Radiation Research, 1989-

L. B. Russell - *Mutation Research*, 1976-
Mouse News Letter, 1987-

G. A. Sega - *Environmental Mutagenesis*, 1985-

D. M. Skinner - *Growth*, 1979-
Gene, 1986-
Physiological Zoology, 1989-

Awards, Honors

R. J. M. Fry, J. B. Storer, and T. J. Mitchell	-	Martin Marietta Energy Systems, Inc., Publication Award, 1989
F. C. Hartman	-	Corporate Fellow, Martin Marietta Energy Systems, Inc., 1989
S. Mitra	-	Fellow, American Association for the Advancement of Science, 1989
S. K. Niyogi	-	Martin Marietta Energy Systems, Inc., Technical Achievement Award, 1989 Scientist of the Year Award, 1989 Martin Marietta Corporation, Thomas Jefferson Cup Winner, 1989
A. L. Olins	-	Honorary Professor, Northeast Normal University, Changchun, PRC, 1988
D. E. Olins	-	Honorary Professor, Northeast Normal University, Changchun, PRC, 1988
R. J. Preston	-	Martin Marietta Energy Systems, Inc., Technical Achievement Award, 1989
A. Stevens	-	Association for Women in Science, East Tennessee Chapter, Annual Award for "Distinguished and Sustained Contributions to Science," 1989
L. C. Waters and C. E. Nix	-	Martin Marietta Energy Systems, Inc., Publication Award, 1989
W. K. Yang	-	National Science Council Visiting Professor, Institute of Biomedical Science, Academia Sinica, Taipei, Taiwan, R.O.C., 1988-1989

Abstracts for Technical Meetings Held October 1, 1988 – September 30, 1989

Ashley, T. G-band position effects on meiotic synapsis and recombination. XVIth International Congress of Genetics, Toronto, Canada, August 20-27, 1988.

Barnett, L. B., R. A. Popp, and S. E. Lewis. The mutagenic impact of X-ray treatment on electrophoretically expressed loci in the mouse. Environmental Mutagen Society Meeting, Cleveland, OH, July 10-17, 1989.

Bhattacharyya, D., W. D. Behnke, and S. Mitra. Reversible unfolding of *E. coli* Ada protein (O^6-methylguanine-DNA methyltransferase). Joint Meeting of the American Society for Cell Biology and the American Society for Biochemistry and Molecular Biology, San Francisco, CA, January 29-February 2, 1989.

Biesiot, P. M., S. Y. Wang, and D. M. Skinner. Tandemly arranged repeat units of a complex G+C-rich satellite have higher methylation levels than copies dispersed in main component DNA. Joint Meeting of the American Society for Cell Biology and the American Society for Biochemistry and Molecular Biology, San Francisco, CA, January 29-February 2, 1989.

Bishop, J. B., W. M. Generoso, M. Katoh, and J. C. Rutledge. Treatment of mouse zygotes with germ cell mutagens results in developmental abnormalities and fetal death. XVIII Annual Meeting, European Environmental Mutagen Society, Drouzhba, Varna, October 3-8, 1988.

Bishop, J. B., M. Katoh, K. T. Cain, and W. M. Generoso. Exposure of female mice to nocodazole one hour after mating results in abnormal chromosome numbers and early death among conceptuses. Nineteenth Environmental Mutagen Society Annual Meeting, Charleston, SC, March 27-31, 1988.

Bishop, J. B., M. Katoh, K. T. Cain, and W. M. Generoso. Exposure of female mice to nocodazole one hour after mating results in abnormal chromosome numbers and early death among conceptuses. XVIII Annual Meeting, European Environmental Mutagen Society, Drouzhba, Varna, October 3-8, 1988.

Bishop, J. B., J. C. Rutledge, and W. M. Generoso. Effects of nocodazole (NOC) and ethylene glycol monomethyl ether (EGMME) on mouse zygotes. Teratology Society Meeting, Richmond, VA, June 3-7, 1989.

Bolch, S. N., and R. A. Popp. Sensitivity of mouse strains to ethylene oxide is due to a single genetic trait. Texas Genetics Society Meeting, San Antonio, TX, March 16-18, 1989.

Bolch, W. E., J. E. Turner, H. Yoshida, K. B. Jacobson, R. N. Hamm, and H. A. Wright. Monte Carlo simulation of indirect damage to biomolecules irradiated in aqueous solution. Tenth Symposium on Microdosimetry, Rome, Italy, May 21-26, 1989.

Byrne, T. E., F. E. Myer, L.-Y. Ch'ang, and W. K. Yang. A determination of the frequency of several retroviral genomic elements in the cDNA from mouse testes. The Tennessee Academy of Sciences Annual Meeting, Cookeville, TN, November 15, 1988.

Cacheiro, N. L. A., C. V. Cornett, and L. B. Russell. Cytogenetic characterization of five Steel translocations in the mouse. The 58th Annual Meeting of the Genetics Society of America, Atlanta, GA, June 30-July 2, 1989.

Cadilla, C. L., K. R. Isham, K.-L. Lee, A. C. Johnson, and F. T. Kenney. A potential insulin regulatory region in a multi-hormonally regulated rat gene. Joint Meeting of the American Society for Cell Biology and the American Society for Biochemistry and Molecular Biology, San Francisco, CA, January 29-February 2, 1989.

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Cosma, G. N., A. C. Marchok, and S. J. Garte. Oncogene activation in cell lines derived from *in vivo* exposure of rat tracheal implants to 7,12-dimethylbenz(a)anthracene (DMBA). American Society for Cancer Research, San Francisco, CA, May 24-27, 1989.

Cosma, G. N., A. C. Marchok, and S. J. Garte. Oncogene activation in rat tracheal epithelial cell lines derived *in vivo* exposure of tracheal implants to DMBA. Meeting on Biology, Toxicology, and Carcinogenesis of Respiratory Epithelium (hosted by Lovelace Toxicology Research Institute), Albuquerque, NM, November 14-16, 1988.

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D'Surney, S. J., and R. A. Popp. Oxygen association - dissociation and stability analysis of hemoglobins with an alpha and beta globin gene mutation in the mouse. The First Biennial Mammalian Developmental Genetics Workshop, Jackson Laboratory, Bar Harbor, ME, September 14-17, 1989.

Engler, D. A., R. K. Matsunami, and S. K. Niyogi. Increased secretion of human epidermal growth factor in E. coli using chloramphenicol. American Association for the Advancement of Science, San Francisco, CA, January 14-19, 1989.

Fry, R. J. M. Experimental radiobiology and radiation protection studies. Annual Meeting of National Council on Radiation Protection & Measurements, Bethesda, MD, April 4-5, 1989.

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Generoso, W. M. Mouse models for understanding human developmental anomalies. Fifth International Conference on Environmental Mutagens, Cleveland, OH, July 10-15, 1989.

Generoso, W. M. Mutagen-induced developmental anomalies in mice. Second Southeast Asian Workshop on Short-Term Assays for Detecting Environmental Mutagens, Carcinogens and Teratogens, Bangkok-Chiang Mai, Thailand, February 6-17, 1989.

Generoso, W. M. Zygote-derived developmental anomalies, a new endpoint of mutagenesis. Twenty-Seventh Hanford Life Sciences Symposium "Multilevel Health Effects Research: From Molecules to Man," Richland, WA, October 18-20, 1988.

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Greer, G., and R. J. Preston. Complementation analysis of X ray sensitive/Ara-C resistant hamster cell (AraC 213) - Human ataxia telangiectasia hybrids. Fifth International Conference on Environmental Mutagens, Cleveland, OH, July 10-15, 1989.

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Hartman, F. C., E. H. Lee, and H. B. Smith. Function of active-site residues of ribulose-bisphosphate carboxylase/oxygenase (RUBISCO). VIIIth International Congress on Photosynthesis, Stockholm, Sweden, August 6-11, 1989.

Hauser, M. R., and J. S. Cook. Independence of ornithine decarboxylase induction and A-system amino acid transport stimulation by the tumor promoter TPA. Federation of American Societies for Experimental Biology, New Orleans, LA, March 19-23, 1989.

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Sega, G. A. Chemical binding to molecular targets in mammalian germ cells and its genetic consequences. Twenty-Seventh Hanford Life Sciences Symposium "Multilevel Health Effects Research: From Molecules to Man," Richland, WA, October 18-20, 1988. **(Extended abstract)**

Sega, G. A. Correlation of DNA and protamine adducts with genetic effects in mammalian germ cells. Workshop on Mutagenicity Risk Assessment, Woods Hole, MA, July 20, 1988.

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Siard, T., K. B. Jacobson, and W. R. Farkas. The effect of queuine on cadmium toxicity in Drosophila melanogaster. Southeastern Chapter of the Society of Toxicology, Augusta, GA, September 14-15, 1989.

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Smith, H. B., and F. C. Hartman. Subtle structural perturbations at the active site of rubisco by concerted site-directed mutagenesis and chemical modification. NATO Advanced Study Institute workshop "Enzymatic and Model Carboxylation and Reduction Reactions for Carbon Dioxide Utilization," Bari (Ginosa), Italy, June 17-28, 1989.

Smith, H. B., F. W. Larimer, and F. C. Hartman. Substitution of Glu-48 of ribulose bisphosphate carboxylase/oxygenase with *S*-carboxymethylcysteine and aspartic acid. Joint Meeting of the American Society for Cell Biology and the American Society for Biochemistry and Molecular Biology, San Francisco, CA, January 29-February 2, 1989.

Soper, T. S., F. W. Larimer, and F. C. Hartman. Replacement of Glu-224 and Lys-226 of ribulose bisphosphate carboxylase/oxygenase from *Rhodospirillum rubrum* by site-directed mutagenesis. Joint Meeting of the American Society for Cell Biology and the American Society for Biochemistry & Molecular Biology, San Francisco, CA, January 29-February 2, 1989.

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Vogt, T., R. Maas, R. Woychik, R. Zeller, and P. Leder. The mouse limb deformity locus: Molecular analysis. Mouse Molecular Genetics, Cold Spring Harbor, NY, August 24-28, 1988.

Wang, S. Y., P. M. Biesiot, and D. M. Skinner. Transcription of a complex satellite DNA. Joint Meeting of the American Society for Cell Biology and the American Society for Biochemistry and Molecular Biology, San Francisco, CA, January 29-February 2, 1989.

Waters, L. C., L.-Y. Ch'ang, and S. J. Kennel. Studies on the expression of insecticide resistance-associated cytochrome P450 in Drosophila using cloned DNA. Seventh International Congress of Pesticide Chemistry, Hamburg, Germany, August 5-10, 1989.

Wawrzyniak, C. J., P. M. Gallagher, M. A. D'Amore, J. E. Carter, S. D. Lund, E. M. Rinchik, and R. E. Ganschow. DNA determinants of structural and regulatory variation within the murine β-glucuronidase gene complex. The 16th Workshop on Molecular and Biochemical Genetics of the Mouse, Park City, UT, April 5-9, 1989.

Woychik, R. P. Insertional mutagenesis in transgenic mice. Second Southeast Asian Workshop on Short-Term Assays for Detecting Environmental Mutagens, Carcinogens, and Teratogens, Bangkok-Chiang Mai, Thailand, February 6-17, 1989.

Woychik, R. P. Insertional mutagenesis in transgenic mice. Twenty-Seventh Hanford Life Sciences Symposium "Multilevel Health Effects Research: From Molecules to Man," Richland, WA, October 18-20, 1988.

Woychik, R. P., B. R. Beatty, and W. L. McKinney, Jr. Insertional mutagenesis in transgenic mice. Twenty-Seventh Hanford Life Sciences Symposium "Multilevel Health Effects Research: From Molecules to Man," Richland, WA, October 18-20, 1988.

Yang, W. K., L. Y. Chang, B. Furkes, F. E. Myer and D. M. Yang. "Insertase" protein of murine type-C retroviruses. Symposium on Structure and Function of Proteins and Nucleic Acids, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, R.O.C., May 11-13, 1989.

Yoshida, H., W. E. Bolch, K. B. Jacobson, and J. E. Turner. Radiolysis of glycylglycine in deoxygenated aqueous solutions. Radiation Research Society 37th Annual Meeting, Seattle, WA, March 18-23, 1989.

Yoshida, H., W. E. Bolch, J. E. Turner, and K. B. Jacobson. The radiation chemistry of glycylglycine in aqueous solutions. Tenth Symposium on Microdosimetry, Rome, Italy, May 21-26, 1989.

Financial Summary and Personnel Distribution

Total Biology Division
FY 1989

Funding Source	Funding in Thousands	Percent of Total Budget	Scientific/ Technical Person-Years*
Department of Energy	10,405	79.3	57.0
National Cancer Institute	452	3.4	3.0
National Heart, Lung and Blood Institute	110	.8	.8
National Institute on Aging	6	.1	-
National Institute of Child Health and Human Development	69	.5	.4
National Institute of Environmental Health Sciences	1,317	10.0	8.9
National Institute of General Medical Sciences	427	3.3	2.5
Department of Agriculture	84	.6	.4
Environmental Protection Agency	55	.4	-
Veterans Administration	8	.1	-
University of Tennessee	135	1.0	1.4
Oregon Health Sciences University	60	.5	.8
	13,128	100.0	75.2

*Does not include ~58 person years: 47 PY Distributed - Administration & clerical, animal caretakers, histology, and kitchen; 11 PY supported by other divisions and ORNL seed money.

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